DUAL FUNCTIONS OF AP-1 IN NEURONAL CELL DEATH AND DIFFERENTIATION

LI LEI

M. Sc. (PUMC&CAMS)

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF MOLECULAR AND CELL BIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2005

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Professor Alan Porter, for providing me the wonderful opportunity to pursue my PhD degree in his laboratory. I am grateful to Alan for his continuous encouragement, support as well as guidance throughout these years.

I am thankful to my graduate supervisory committee, Drs. Victor Yu and Edward Manser for their constructive suggestions and critical comments.

I would also like to thank past and present members of the AGP laboratory for their helpful discussion, technique assistance, cooperation and friendship. Especially thanks go to Dr. Zhiwei Feng for his patience, guidance as well as helpful suggestions.

I thank all members in VY lab for idea sharing at our Apoptosis Club.

I appreciate very much the friendship with Dr. Tong Zhang, Dr. Jormay Lim and Dr. Zhihong Zhou. The wonderful time we spend together in IMCB will be in my mind forever.

My heartful appreciation goes to my beloved parents for their constant support and encouragement, without whom this would have remained but a dream. Finally, my deepest gratitude goes to my husband for his unconditional love, understanding and warm support through the years.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AP-1	activator protein 1
ATP	adenosine 5’ - triphosphate
bZIP	basic-region leucine zipper
Caspase	cysteine-dependent aspartate-specific proteinase
CDK	cyclin-dependent kinase
CGA/B	chromogranin A/B
cGMP	cyclic GMP
CNS	central nervous system
Cox-2	cyclooxygenase-2
DN	dominant negative
ERK	extracellular signal regulated kinase
FADD	Fas-associated death domain
GSK-3	glycogen synthase kinase-3
HO-1	heme oxygenase-1
HSP70	heat shock protein 70
JAK	Janus kinase
JNK	Jun N-terminal kinase
IL	interleukin
MAPK	mitogen activated protein kinase
MEK1	MAP/ERK kinase
Mn-SOD	Mn^{2+}-dependent superoxide dismutase
NAD(H)	nicotinamide adenine dinucleotide
NCAM	neural cell adhesion molecule

NGF	nerve growth factor
NMDAR	N-methyl-D-aspartate (NMDA) receptor
NO	nitric oxide
NOS	nitric oxide synthase
PAGE	polyacrylamide gel electrophoresis
Rb	retinoblastoma
SD	standard deviation
SDS	sodium dodecyl sulfate
SgII	secretogranin II
SIN-1	3-morpholinosydnonimine
SNP	sodium nitroprusside
TAM-67	transactivation mutant-67
TNFR	tumor necrosis factor receptor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRADD	TNFR-associated death domain

LIST OF PUBLICATIONS

Lei Li, Alan G. Porter (2005). c-Jun/AP-1 Regulates Secretogranin II, a New Class of Protein that Mediates Neuronal Differentiation and Protection from Nitric Oxide-Induced Apoptosis.

J. Biol. Chem. Under revision.

Lei Li, Zhiwei Feng, and Alan G. Porter (2004). JNK-dependent Phosphorylation of c-Jun on Serine 63 Mediates Nitric Oxide-induced Apoptosis of Neuroblastoma Cells.

J. Biol. Chem. 279, 4058-4065.

Zhiwei Feng, **Lei Li,** Poh Yong Ng, and Alan G. Porter (2002). Neuronal Differentiation and Protection from Nitric Oxide-Induced Apoptosis Require c-Jun-Dependent Expression of NCAM140.

Mol. Cell. Biol. 22, 5357-5366.

SUMMARY

Transcription factors in the AP-1 family (which includes c-Jun) play critical roles in basal CNS function and in patho-physiology associated with neuronal disorders. Nitric oxide (NO) overproduction is partly responsible for neuronal cell death in various types of neurodegeneration. An involvement of AP-1 in NO-induced neuronal apoptosis has not been explored. I found that in human SH-Sy5y neuroblastoma cells, NO induced apoptosis following JNK activation and phosphorylation of c-Jun almost exclusively on Ser-63. NO-induced apoptosis was inhibited in cells stably transformed with dominant-negative c-Jun in which Ser-63 is mutated to alanine (S63A), but not in cells transformed with dominant-negative c-Jun (S73A). Ser-63 of c-Jun (but not Ser-73) was required for NO-induced, c-Jun-dependent transcriptional activity. NO-induced apoptosis and Ser-63 phosphorylation of c-Jun were inhibited in SH-Sy5y cells transformed with dominant-negative *jnk*. I conclude that NO-inducible apoptosis is mediated by JNK-dependent Ser-63 phosphorylation of c-Jun in neuroblastoma cells.

Opposite observations were made using another dominant negative form of c-Jun, TAM67 (transactivation domain deletion mutant of c-Jun). Cells stably over-expressing TAM67 were sensitized to NO, suggesting a protective role of c-Jun/AP-1. Microarray analysis identified secretogranin II (SgII) as an NO-inducible, c-Jun-regulated protective gene. NO stimulated reporter gene expression from a short *sgII* promoter region harboring its own intact CRE element (but not a mutated CRE element) in transiently transfected SH-Sy5y neuroblastoma cells. Basal and NO-inducible expression of the *sgII* gene, as well as basal SgII protein synthesis were severely compromised in TAM67 stable cells, which were more sensitive to NO-induced apoptosis and failed to undergo nerve growth factor (NGF)-dependent

neuronal differentiation. When *sgII* mRNA was stably transformed into TAM67 cells, neuronal differentiation and resistance to NO were restored. RNA interference-mediated *sgII* knockdown rendered SH-Sy5y cells sensitive to NO-induced apoptosis and abolished neuronal differentiation. Thus, SgII synthesis largely depends on c-Jun/AP-1-mediated transcription. Importantly, SgII represents a new class of proteins that counteracts NO toxicity and mediates NGF-induced neuronal differentiation of neuroblastoma cells.

The opposing effects of the dominant-negative c-Jun (TAM-67) and S63A can be explained: TAM-67 efficiently inhibits constitutive AP-1-mediated transcription in SH-Sy5y cells and thus blocks SgII-mediated cell survival. The synthesis of SgII does not require Ser-63 phosphorylation of c-Jun, because it occurred in the absence of NO stimulation. In contrast to TAM-67 cells, SgII proteins were still synthesized at normal levels in NO-resistant S63A cells, indicating the c-Jun/AP-1-dependent SgII survival pathway is intact in these cells. The S63A construct blocked the pro-apoptotic JNK-c-Jun pathway without affecting the synthesis of neuroprotective SgII, so the cells were resistant to apoptosis compared to SH-Sy5y cells and TAM-67 cells. The basal activity of c-Jun/AP-1 factor(s) (independent of c-Jun phosphorylation on Ser-63) is able to counteract relatively low levels of NO - in part through the constitutive expression of neuroprotective SgII. In contrast, a threshold toxic concentration of NO will lead to c-Jun phosphorylation on Ser-63 by JNK that triggers apoptosis *via* yet to be discovered c-Jun targets.

CHAPTER 1 INTRODUCTION

This chapter will begin with an overview of programmed cell death (apoptosis), followed by two mini reviews on nitric oxide and AP-1, respectively. Emphasis will be placed on the current understanding of nitric oxide chemistry and its biological significance. In the mini review of AP-1, the regulation of AP-1 activity and the overall roles of AP-1 are discussed; and molecular mechanisms mediating AP-1 functions in different processes will be highlighted. Lastly, the rationale of the thesis will be discussed.

1.1 Programmed cell death

Programmed cell death (PCD) is an evolutionary conserved process that is important for multicellular organisms to delete unwanted cells during development and homeostasis (Ellis *et al*., 1991; Jacobson *et al*., 1997). Two major types PCD were characterized so far: apoptosis (type I) and autophagic cell death (type II).

Morphologically, cells undergoing apoptosis exhibit membrane blebbing, cytoplasmic shrinkage and chromatin condensation. Apoptotic cells are degraded into membrane-bound fragments called apoptotic bodies, which are rapidly engulfed by the neighboring cells or professional macrophages (Kerr *et al*., 1972). The process of apoptosis is neat and quick with no induction of inflammation, which may be one reason why it was neglected for so long. In contrast, necrosis, a pathological form of cell death resulting from acute cellular injury, is characterized by cell swelling and lysis, release of cytoplasmic contents, and the induction of an inflammatory response (Wyllie *et al*., 1980). Biochemically, apoptotic cells exhibit externalization of phosphatidylserine (PS), reduction of mitochondrial transmembrane potential, release of cytochrome *c* from mitochondria into the cytoplasm, degradation of chromosomal

DNA into oligonucleosomal fragments and selective cleavage of a subset of intracellular proteins (see reviews by Fadok *et al*., 1998; Green and Reed, 1998; Stroh and Schulze-Osthoff, 1998).

The mechanisms of how apoptosis is initiated and executed remained unclear until the molecular identification of the key components of this intracellular suicide program. The typical apoptotic process can be divided into three functional distinct phases: an induction phase, during which the cell is challenged by changes in the cellular environment and the nature of which depends on the specific death-inducing signals; an effector phase, during which the central executioners are activated and the cells become committed to die; and a degradation phase, during which cells acquire the biochemical and morphological features of end-stage apoptosis (Green and Kroemer, 1998; Wilson, 1998). Genetic studies of the nematode worm *Caenorhabditis elegans* have provided powerful clues to the identity of the molecular species important in controlling apoptosis (Wilson, 1998). These studies identified three *C. elegans* death genes, named *egl-1*, *ced-3* and *ced-4* (egl, egg-laying abnormal; ced, cell death abnormal), that were required for developmental apoptosis (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986), and a fourth gene, *ced-9*, that inhibits apoptosis (Hengartner *et al*., 1992). The molecular cloning of *egl-1*, *ced-3*, *ced-4* and *ced-9* led to the finding that these core components of the cell death machinery in *C. elegans* have counterparts in other organisms including mammals (Fig 1.1). The *ced-3* gene encodes a protein similar to the cysteine protease interleukin-1β-converting enzyme (ICE) (Yuan *et al*., 1993), a prototype of a family of proteases (collectively called caspases). The *ced-4* gene encodes a protein similar to a mammalian protein celled Apaf-1 (Apaf, apoptotic protease-activating factor) (Zou *et al*., 1997). The *ced-9* gene encodes a protein similar to the mammalian protein Bcl-2 (Bcl, B cell lymphoma) (Hengartner and Horvitz,

1994), a prototype of a family of both antiapoptotic and proapoptotic proteins. Lastly, the *egl-1* gene encodes a protein similar to the mammalian "BH3-only" (BH, Bcl-2 homology domain) proteins, a subfamily of Bcl-2 family. The fact that these key cell death components in *C. elegans* have mammalian counterparts indicates that the molecular mechanism of PCD is evolutionarily conserved (Steller, 1995).

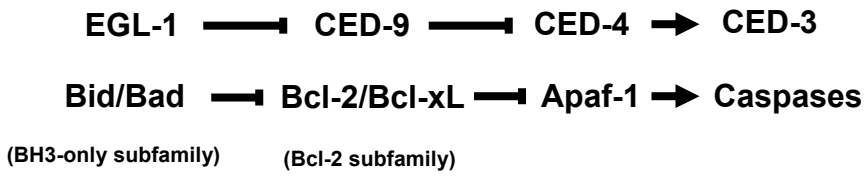

Fig 1.1 Core cell death components in *C. elegans* and their counterparts in mammals.

Among the aforementioned three major players in apoptosis: caspases, Bcl-2 family proteins and Apaf-1 adaptor proteins, caspases are the central executioners (Hengartner, 2000). Most of the morphological changes of apoptotic cells are probably caused by caspase activation and consequent cleavage of their substrates that fulfill important cellular functions (Hengartner, 2000; Raff, 1998). The other two players function through directly or indirectly regulating caspase activity. There are two general pathways leading to caspase activation and cell death. Death signals originating from the death receptors (TNFR or Fas/CD95/Apo1) or resulting in mitochondrial dysfunction trigger the activation of caspase-8 or caspase-9 respectively through their individual adaptor proteins FADD/TRADD or Apaf-1. These activated initiator caspases in turn activate the downstream executioner caspases 3, 6 or 7 which further cleave a variety of cellular proteins leading to the dismantling of the nucleus, DNA degradation, cytoskeleton breakdown, and detachment of the cells from their

neighbours. The cross talk between the two pathways is mediated by Bid, which can translocate to mitochondria upon cleavage by caspase-8 and activate the mitochondria-mediated cell death pathway (Adams and Cory, 1998; Colussi and Kumar, 1999; Slee *et al*., 1999). A schematic stepwise representation for caspase activation during apoptosis is illustrated in Fig 1.2.

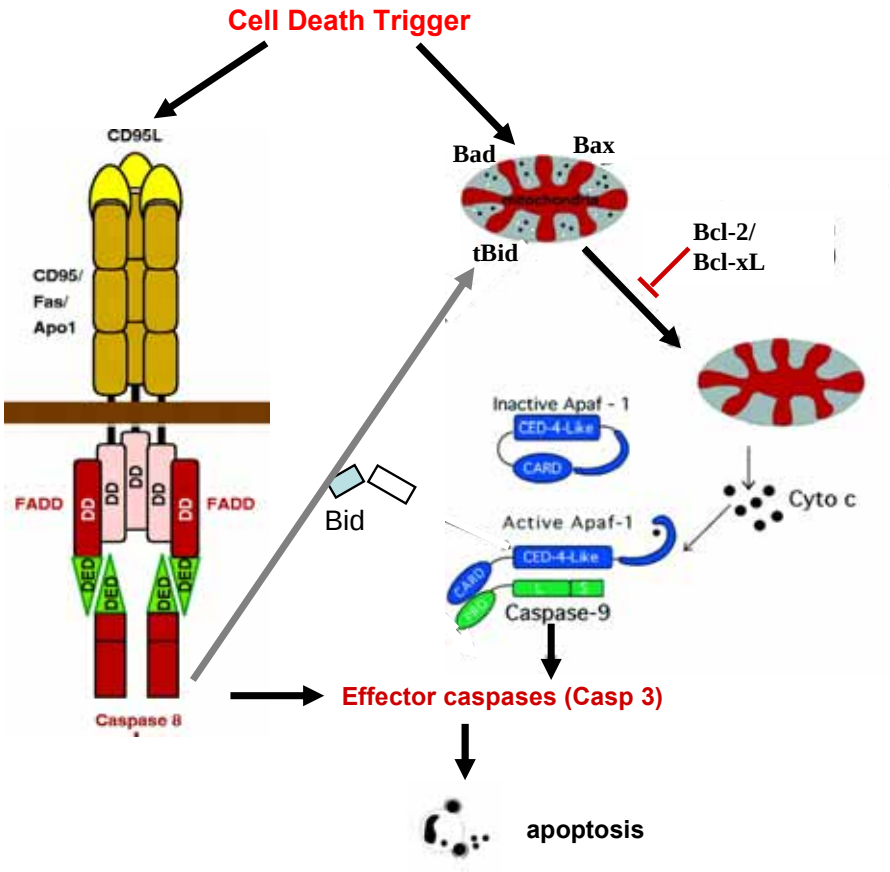

Fig 1.2 Two classical pathways leading to caspase activation. Death receptor engagement causes the activation of initiator caspase-8. Mitochondrial damage results in cyto C release and intiator caspase-9 activation. In both cases, the initiator caspases further activate the effector caspases leading to cellular proteins cleavage.

In contrast to the condensation prominent apoptosis, the autophagic cell death is autophagy prominent characterized by formation of autophagic vacuoles, degradation of cytoplasmic components including Golgi apparatus, polyribosomes, and endoplasmic reticulum due to activation of proteases in lysosomes. Intermediate and microfilaments are largely preserved, probably because cytoskeleton structure is important for autophagocytosis. Cell death of this type is independent of caspases and DNA fragmentation was rarely seen. In many tissues, autophagy is a means of reducing cell mass prior to apoptosis. It can also be used in situation in which conventional apoptosis pathways are blocked or limited (Bursch, 2001; Lockshin and Zakeri, 2004).

Although distinct biochemical and molecular features have been be assigned to these two different types of PCD, they are not mutually exclusive phenomena (Bursch, 2001). Apoptosis can start with autophagy and autophagy can end with apoptosis. Furthermore, blockage of caspases can result in a cell to default to autophagic cell death from apoptosis.

The occurrence of PCD may not be an essential event in the *C. elegans* lifespan since the worm appears normal in size and viability with total elimination of apoptosis (Raff, 1998). However, PCD plays a vital role in more complex organisms, enabling the normal development of the organisms (Jacobson *et al*., 1997), maintaining tissue homeostasis in the adults as well as keeping the immune system effective (Raff, 1998). Deregulation of PCD has been associated with various human diseases (Thompson, 1995). For example, there are many disorders where cells die prematurely: heart cells die during a heart attack and brain cells die during a stroke (Raff, 1998). In these acute conditions, many cells die by necrosis. But some of the less badly damaged cells die by apoptosis. Also, in neurodegenerative diseases, such as Alzheimer's, Parkinson's or

Huntington's, nerve cell loss occurs slowly. It has been established that caspase activity is involved in the processes leading to the pathology of these diseases (Pettmann and Henderson, 1998; Yuan and Yankner, 1999). Insufficient cell death is linked to the occurrence of cancer and autoimmune diseases. In the former case, the malignant cells with genetic mutations escape the cellular guardian systems and divide, causing tumorigenesis and hence malignancy. In the latter case, deficient cell death in the immune system results in prolonged or overactive immune responses. A further understanding of the molecular genetic mechanisms underlying those diseases is expected to provide clues for more specific therapies.

1.2 Nitric oxide in health and disease

1.2.1 Formation and chemistry of nitric oxide

Nitric oxide (NO) is catalytically produced by 3 different NO-synthase (NOS) isoforms in a reaction scheme (Brune *et al*., 1998; Stuehr, 1999), involving the five electron oxidation of the terminal guanido nitrogen of the amino acid L-arginine to form NO and L-citrulline (Fig 1.3). In addition, NO can also be generated non-enzymatically in tissues by either direct disproportionation or reduction of nitrite to NO under the acidic and highly reduced conditions which occur in disease states, such as ischemia (Zweier *et al*., 1999).

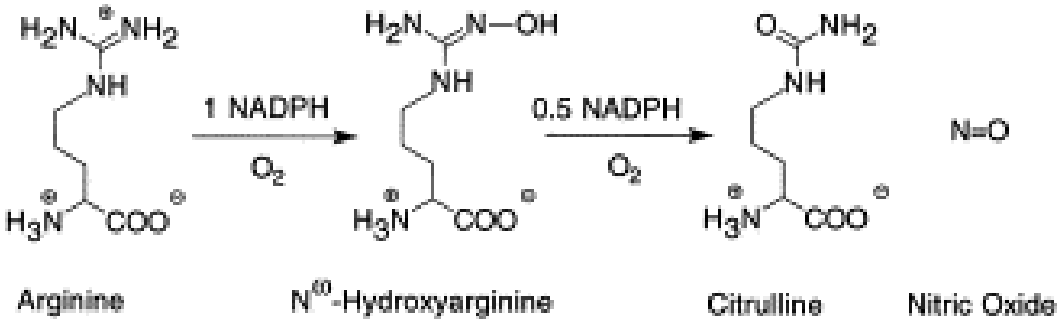

Fig 1.3 Enzyme-catalyzed formation of NO from L-Arginine. Hydroxylation of L-Arginine generates N-hydroxy-L-Arg (NOHarg) as an intermediate. The second step converts NOHarg to NO and citrulline (Stuehr, 1999).

The low output NO (also known as signal molecule NO) generation by constitutively expressed NOS (cNOS) can last for only short periods (seconds to minutes) and mediates homeostatic processes such as neurotransmission and blood pressure regulation (Nathan and Xie, 1994a). cNOS is further classified as eNOS or

nNOS according to the characteristic cells expressing them (Nathan and Xie, 1994a; Nathan and Xie, 1994b). Elevated intracellular Ca^{2+} concentrations seem important for the full enzyme activity of cNOS. For example, in the CNS, acute neuronal damage is coupled to glutamate release into the extracellular matrix, membrane-spanning NMDAR activation and consequent Ca^{2+} influx into the cellular compartment, which greatly enhances the eNOS activity and causes massive NO generation and cytotoxicity. The high output NO (also known as killer molecule NO) is synthesized predominantly by inducible NOS (iNOS) (Nathan and Xie, 1994a) and can last for long periods (hours to days). Although the iNOS activity is independent of Ca^{2+}, its expression is highly inducible upon stimulation of cells by microbes and microbial products, some tumor cells and numerous cytokines (Nathan and Xie, 1994a; Nathan and Xie, 1994b). NO generation in such scenari often correlates with non-specific host defense *via* infection or inflammation. Besides, other factors like intracellular localization of NOS, palmitoylation and phosphorylation of NOS are believed to modulate NOS enzyme activity (Nathan and Xie, 1994a; Nathan and Xie, 1994b). Recently, mitochondrial localization of NOS has also been proposed (Ghafourifar and Richter, 1997; Giulivi *et al*., 1998; Kanai *et al*., 2001), the importance of which will be discussed in section 1.2.2. In the laboratory, to mimic NO generation irrespective of NOS involvement, NO releasing compounds (also called "NO donors") are valuable tools (Butler *et al*., 1995). NO donors preserve NO in their molecular structure and evoke biological activities upon decomposition (Brune *et al*., 1998). Examples are organic nitrate such as sodium nitroprusside (SNP), 3-morpholino-sydnonimine (SIN-1) and diethylenetriamine nitric oxide adduct (DETA-NO). Once generated, NO is highly diffusible and easily passes membranes and sets up trans-cellular or local concentration gradients within cells and subcellular compartments like mitochondria (Boyd and

Cadenas, 2002). The steady-state level of NO will be determined by the nature of the local microenviroment (discussed below).

Although NO is a radical, it lacks the reactivity normally inherent to other radicals. This makes NO relatively innocuous to cells, but some key chemical reactions can lead to the production of more reactive species, potentially more toxic than NO itself. Biologically significant NO redox and additive reactions include those with (di)oxygen and its various redox forms and with transition metals (Boyd and Cadenas, 2002; Cooper, 1999; Torres and Wilson, 1999) as summarized in Fig 1.4.

Oxidation of NO by O_2 alone can lead to various nitrogen oxide species (collectively called NO_X) existing simultaneously in aqueous solution including NO, $^{\bullet}OONO$, NO_2, $(NO)_2$, N_2O_3, N_2O_4, NO_2^- and NO_3^- (irrespective of the redox reactions with other biochemical components in the microenviroment) (Boyd and Cadenas, 2002). In addition, NO can undergo radical-radical interactions with other oxygen- and nitrogen-centered radicals. The former includes its reaction with $O_2^{\bullet-}$ to yield peroxynitrite ($ONOO^-$); with the hydroxyl radical ($HO^{\bullet}$) to yield HNO_2; and with the peroxyl radical ($ROO^{\bullet}$) to yield ROONO. The latter includes its reaction with NO_2 to yield N_2O_3; with $ONOO^-$ to yield nitrosating species (Boyd and Cadenas, 2002). So the nature of NO_X can be significantly altered by the availability of other oxyradicals which are normally ubiquitous and highly diffusible in the cytosol. Alternatively, the fate of NO can be shifted if it is produced by NOS in close proximity to the sources of $O_2^{\bullet-}$ or H_2O_2 (such as NADPH oxidase) (Fig 1.4).

Of the entire NO related species, NO^-, NO^+ and $ONOO^-$ are of great biological significance because of their reactivity with components in the cellular microenvironments.

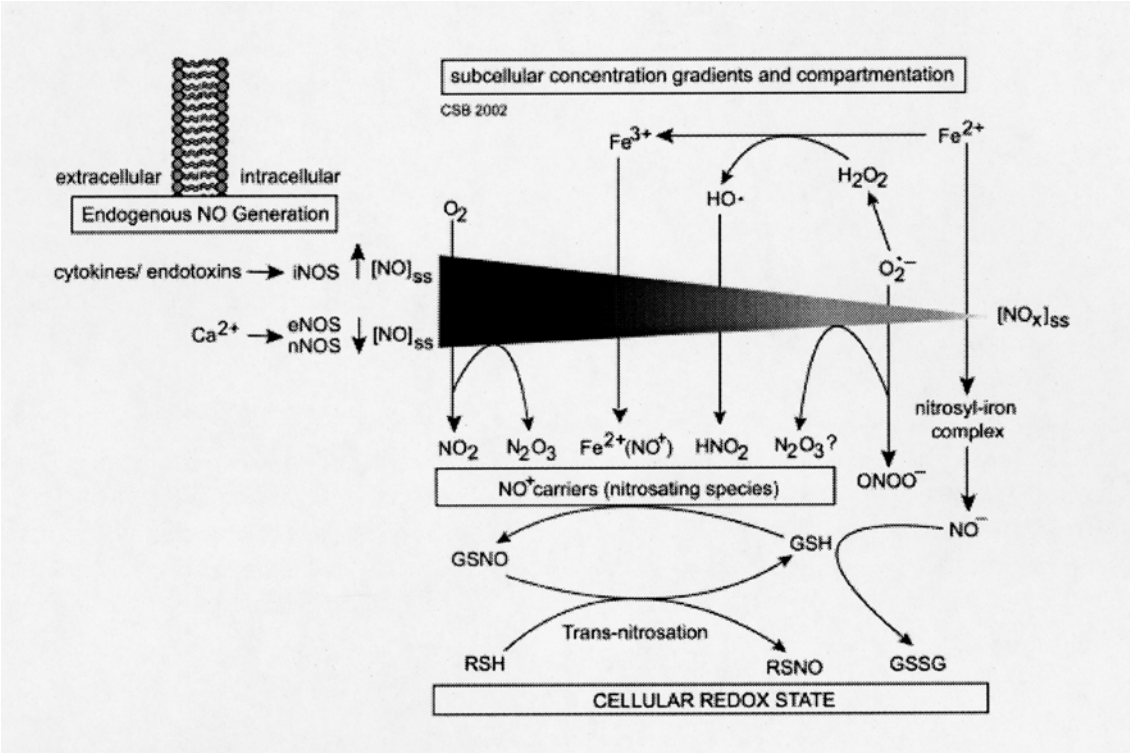

Fig 1.4 NO chemistry in the cellular environments (Boyd and Cadenas, 2002). Endogenous NO is synthesized by different NOS enzymes. Once generated, NO freely diffuses creating concentrations across the subcellular compartments. Redox or additive reaction with constituents of the local microenviroment converts NO to a number of NO_x species and establishes the steady-state concentrations of each. The generation of NO+ carriers (nitrosating species) is likely under physiological conditions, leading to the formation of GSNO and RSNO species, which participate in trans-nitrosation reactions. The ratio GSH/GSSG, a key index of the cellular redox potential, can be shifted in opposite directions by either NO^+ carriers or NO^-, in turn establishing the importance of the nature of the NO_x species to the redox state.

NO^+, the oxidized form of NO (by electron loss), has the potential to regulate cell signaling pathways due to its ability to nitrosate (Gaston, 1999; Hughes, 1999). Nitrosation is a process in which the NO^+ group is transferred (usually from a carrier compound such as N_2O_3 and metal-nitrosyl complexes) to a nucleophilic center, often

to a sulfur or nitrogen lone pair of electrons. These reactions create a series of new NO^+ donors like *N*-nitrosamines and *S*-nitrosothiols, which can then participate in further trans-nitrosation reactions. *S*-nitrosothiol formation, through the S-nitrosation of either free or protein thiols, has real biological significance (it will be discussed in section 1.2.3). *S*-nitrosothiols act as a bioactive pool serving as a source and sink of NO, buffering the free NO. *S*-nitrosothiols are relatively stable, prolonging the half-life of NO and protecting against the generation of more toxic NO_X species (Boyd and Cadenas, 2002). Furthermore *S*-nitrosation of proteins, occuring favorably under physiological conditions, is reversible and capable of trans-nitrosation reactions: two criteria that point to S-nitrosation as a potential cellular regulatory mechanism (Fig 1.4). In this regard, reduced glutathione (GSH), the most abundant cellular thiol, is likely to be the major intracellular mediator of NO storage and transport, forming *S*-nitrosoglutathione (GSNO). It is noteworthy that *S*-nitrosothiol formation is considered the typical redox-based NO-signaling mechanism, which is cGMP-independent and has previously been considered to mainly account for the cytostatic, cytotoxic or protective NO effects (Lipton, 1999).

NO^-, the reduced form of NO (by electron gain), is a short-lived species in solution, decomposing via dimerization and dehydration to give nitrous oxide. NO^- reacts with a variety of targets such as iron-sulfur center of cytochrome *d*, cysteine residue of ferrocytochrome *c* and more importantly, it reacts with oxygen to generate peroxynitrite (Hughes, 1999). That is at least partly responsible for its toxic effects.

$ONOO^-$ is a highly reactive product of NO. $ONOO^-$ can react with tyrosine residues in many target proteins via nitration and also it involves in the process of hydroxylation. Furthermore, peroxynitrite is a strong oxidizing agent and readily oxidizes most components in mitochondria, causing oxidation and cross-linking of

proteins, irreversible inhibition of most of the mitochondrial complexes, oxidation of non-protein thiols, membrane lipids and thus disrupt the mitochondrial membrane (Brown, 1999; Hughes, 1999; Radi *et al*., 2002).

NO also reacts with transition metal such as iron or copper and regulate the cell signaling pathways. NO is capable of binding to both the ferric (Fe^{III}) and ferrous (Fe^{II}) oxidation states of iron (Boyd and Cadenas, 2002; Cooper, 1999). The reaction with Fe^{III} involves a catalytic process called reductive nitrosation: reduction of metal by NO and formation of bound NO^+, the nitrosonium cation. Reduction of non-heme iron has also been observed, including iron-sulfur centers in proteins such as components of the mitochondrial respiratory chain and other mitochondrial enzymes. NO also shows high affinity to Fe^{II}, forming a stable nitrosyl complex in competion with O_2. NO in such a process is reduced to the nitroxyl anion (NO^-), which can oxidize sulfhydryl (thiol) groups. Oxymyoglobin and hemoglobin are important NO scavengers in this regard (Fukuto, 1995). NO also can react with copper (Cu^{2+}) proteins (Torres and Wilson, 1999), acting as a fast one-electron reductant at Cu^{2+}, such as haemocyanin, tyrosinase and cytochrome *c* oxidase.

Based on the NO chemistry discussed above, formation of NO-derived species shows high dependence on pH, O_2 tension, redox state and the transition metal content of the local environment (Boyd and Cadenas, 2002). The steady-state levels of oxygen- and nitrogen-centered radicals are a key contributor to the oxidative potential, whereas intracellular GSH is a major determinant of the reductive potential (Fig 1.4). The net balance between the oxidative and reductive potentials within a cell determines the cellular redox state and perhaps the fate of the cell or organism.

1.2.2 Influence of nitric oxide on important cellular organelles

The mitochondria is a primary intracellular target of NO (Radi *et al*., 2002) for the following reasons: (1) the diffusible nature of NO through biomembrane as well as local generation of NO in mitochondria; (2) the presence of large amounts of metalloproteins such as cytochrome *c* oxidase, which rapidly react with NO; (3) the intramitochondrial thiol pool, which serves as a primary reactant for the actions of oxidized forms of NO such as N_2O_3 that lead to S-nitrosation; and (4) the intramitochondrial formation of $O_2^{\cdot-}$ (Boyd and Cadenas, 2002) that results in rapid NO consumption and formation of the strong oxidant and nitrating species peroxynitrite anion ($ONOO^-$).

NO impairs electron flux through the respiratory chain through the inhibition of multiple sites (Boyd and Cadenas, 2002; Brown, 1999; Radi *et al*., 2002). At low physiological concentrations (10 nM – 1 μM), NO inhibits cytochrome *c* oxidase (Complex IV, the terminal complex of the mitochondrial respiratory chain, responsible for about 90% of oxygen consumption and for virtually all energy production in cells.) and the ubiquinone-bc_1 segment (complex III) of the respiratory chain. The inhibition of cytochrome *c* oxidase is reversible and involves oxidation of the heme group (O_2 binding domain) of cytochrome aa_3 and possibly Cu_B^+ through the competitive binding with O_2. The interactions of NO with cytochrome *c* oxidase, precluding the reduction of molecular oxygen, lead to a larger concentration of reduced components of the electron transport chain such as $O_2^{\cdot-}$, NO^- and NO_2^- (Radi *et al*., 2002). The inhibition of the bc_1 segment is partially reversible and leads to the auto-oxidation of ubisemiquinone with the subsequent generation of $O_2^{\cdot-}$ and thus H_2O_2. At higher concentrations (>1 μM), NO can directly oxidize ubiquinol, promoting ubisemiquinone auto-oxidation. The fate of $O_2^{\cdot-}$ generated in these reactions is highly

dependent on the local NO concentrations: at a low $[NO]_{ss}$ superoxide dismutation to H_2O_2 is favored; and at a high $[NO]_{ss}$ the conversion to $ONOO^-$ is favored. Peroxynitrite itself can oxidize ubiquinol, amplifying generation of $O_2^{\bullet -}$ as well as itself potentially. The NO-induced production of $O_2^{\bullet -}$ and $ONOO^-$ results in the selective and persistent inhibition of NADH:ubiquinone reductase (Complex I) and Complex II activity. Peroxynitrite also causes irreversible damage to mitochondrial ATP synthase (Complex V), presumably through the oxidation of critical thiols, and promotes the permeability transition, cytochrome *c* release and apoptosis. The targeting sites of NO as well as peroxynitrite on mithchondria is illustrated in Fig 1.5.

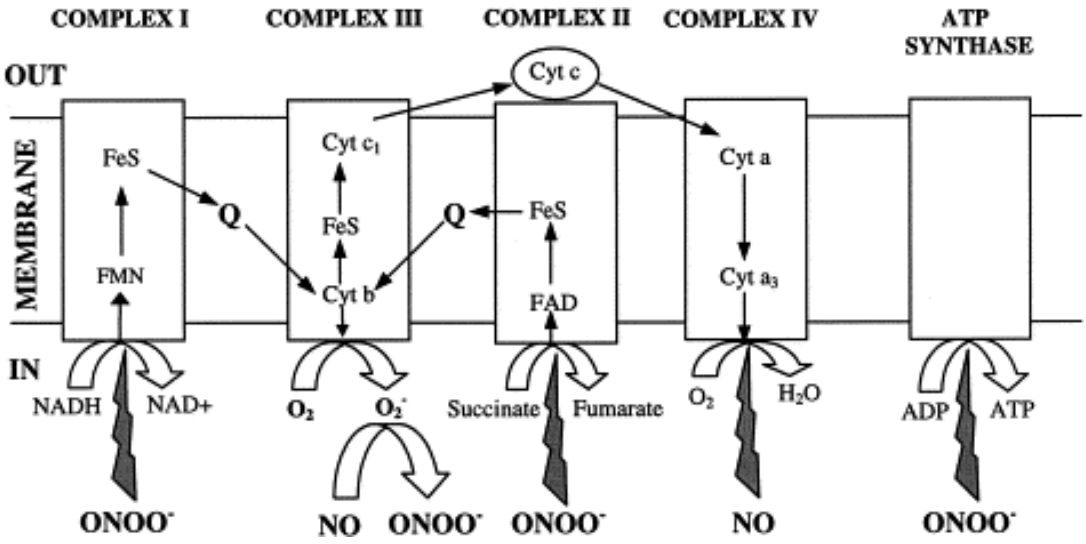

Fig 1.5 The targeting sites of NO and $ONOO^-$ on the mitochondria complexes (Brown, 1999). Electron pathways are represented by light arrows, and inhibitions are depicted as thunderbolts.

Besides the components of respiratory chain, NO and its derivatives also readily react with mitochondrial matrix proteins including aconitase, MnSOD and creatine

kinase (Radi *et al*., 2002). NO influence on cell energy metabolism is partially due to the NO-dependent disruption of the Fe-S cluster present in the active site of mitochondrial aconitase since it participates in the Krebs cycle. NO can bind to the iron center and reversibly inhibit aconitase at low rates while $ONOO^-$ can rapidly oxidize and disrupt the cluster. MnSOD, the critical enzyme for detoxification of intramitochondrial $O_2^{\bullet -}$, is inactivated by $ONOO^-$-mediated nitration of critical Tyr-34. In turn, inactivation of MnSOD promotes a vicious cycle resulting from an enhanced mitochondrial steady-state concentration of $O_2^{\bullet -}$ which would favor further $ONOO^-$ formation and mitochondrial oxidative damage. Mitochondrial creatine kinase, the enzyme for synthesis and degradation of creatine-phosphate, is inactivated by $ONOO^-$ through oxidation of critical protein thiols. Inactivation of creatine kinase may affect mitochondrial energy metabolism and indirectly promote calcium accumulation in the cytosol due to the alterations of the ATP-dependent calcium transport to the mitochondria.

NO interactions with cytochrome *c* oxidase may play two important physiological roles (Radi *et al*., 2002). Firstly, low levels of NO may serve as a physiological regulator of tissue respiration: it causes a transient, reversible de-energization of the mitochondria and help to attenuate the oxygen tension gradient. The physiological significance for such a regulation is not completely understood, although it has been proven from both exogenously added NO and endogenously produced NO on various cell types (Brown, 1999). Since NO competes with oxygen at cytochrome *c* oxidase, some scholars (Brown, 1995) suggest that NO may be a physiological regulator of the oxygen sensitivity of respiration in tissues. Secondly, NO interactions with cytochrome *c* oxidase may exert anti-apoptotic actions through inhibiting mitochondrial signaling of cell death in the following way: interaction

between NO and cytochrome *c* oxidase results in mitochondrial depolarization, inhibition of calcium uptake, blockage of the mitochondrial transition pore (MTP) opening and thus hold up the release of pro-apoptotic components such as cytochrome *c* and Diablo/Smac. NOS localization at the inner membrane of mitochondria places a source of NO generation adjacent to the respiratory chain (Boyd and Cadenas, 2002), which indicates that NO would function to regulate the mitochondrial respiration physiologically based on the above discussion.

In contrast, long-time exposure to NO (most probably through $ONOO^-$) can lead to the irreversible inhibition of mitochondrial respiration chain and persistent blockage of ATP synthesis (Boyd and Cadenas, 2002; Radi *et al*., 2002). A transient drop in ATP levels appeared to correlate with apoptosis, an energy-dependent process; whereas a persistent or complete decline in ATP results in necrosis. In addition, high NO fluxes or $ONOO^-$ induce the permeability transition and, cytochrome *c* and Ca^{2+} release from mitochondria and apoptosis. Moreover, release of cytochrome *c* is associated with a burst of mitochondrial $O_2^{\bullet -}$ generation that can further alter the cellular redox state. The action of NO and peroxynitrite and respective consequences for mithchondrial respiration is summarized in Fig 1.6.

The nucleus is another cellular target for NO. NO has been shown to cause G:C → A:T transitions and to mediate DNA strand breaks (Kroncke *et al*., 1997). *N*-nitrosation of deoxynucleotides and DNA bases deamination predominantly account for both cases. Indirect induction of DNA strand breaks, such as via *N*-nitrosamine formation and subsequent alkylation reactions, via activation or inhibition of enzymes necessary for nuclear homeostasis, is also possible. Since DNA damage is potentially hazardous for the cell, being able to cause mutation and transcription or translation inhibition, there are a variety of mechanisms for repair in the cell. p53 and PARP are

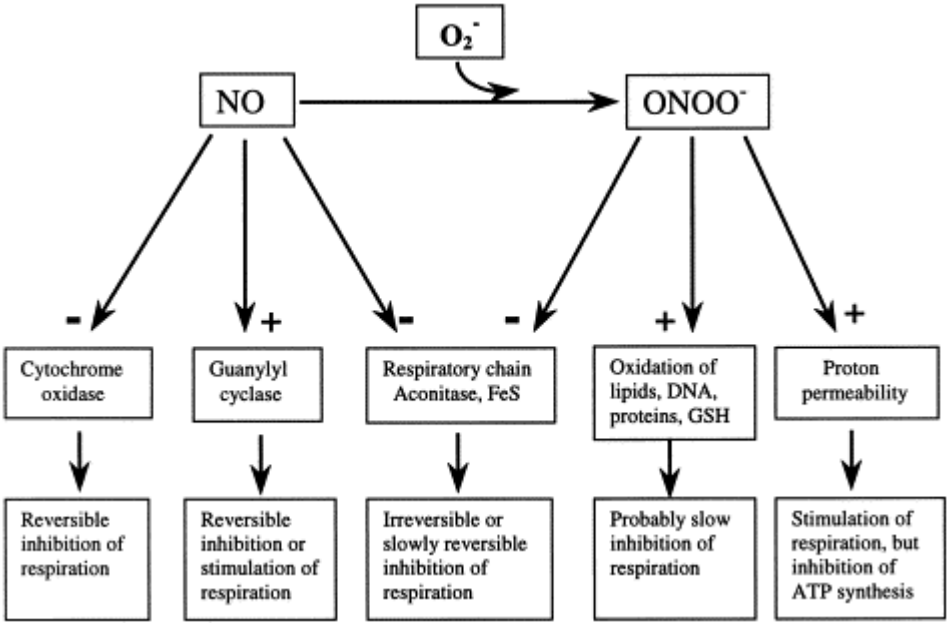

Fig 1.6 The actions of NO and $ONOO^-$ on mitochondria and the consequences (Brown, 1999).

two proteins known to be involved in such repair. p53 is known as a "guardian of the genome" and subject to quick upregulation at the protein level upon DNA damage (Chernova *et al*., 1995; Enoch and Norbury, 1995). It can induce G1 arrest through transcriptional activation of p21, an inhibitor of cyclin dependent kinases, and thereby block the progression of the cell cycle and permit DNA repair. PARP [poly (ADP-ribose) polymerase] is a constitutively expressed nuclear protein which is regarded as a molecular nick sensor and has a functional role during rejoining of DNA strand breaks (Kroncke *et al*., 1997). After binding to DNA breaks, PARP automodifies itself by adding several branched polymer chains of up to 200 ADP-ribose residues, each resulting in PARP inhibition and causing its dissociation from the DNA strand breaks. The poly (ADP-ribose) polymers are degraded later. PARP is proposed to function in the following ways: it may protect DNA strand breaks during early stages of

recombination and repair, may induce cell cycle arrest by transiently blocking DNA replication or may simply provide an emergency signal. NO treatment has been shown to induce p53 expression and to activate PARP in different cells. While p53 expression is not detrimental to the cells, activation and consequent poly(ADP-ribosylation) of PARP lead to a severe cellular depletion of ATP and NAD^+, which may contribute to cell death (Fig 1.7).

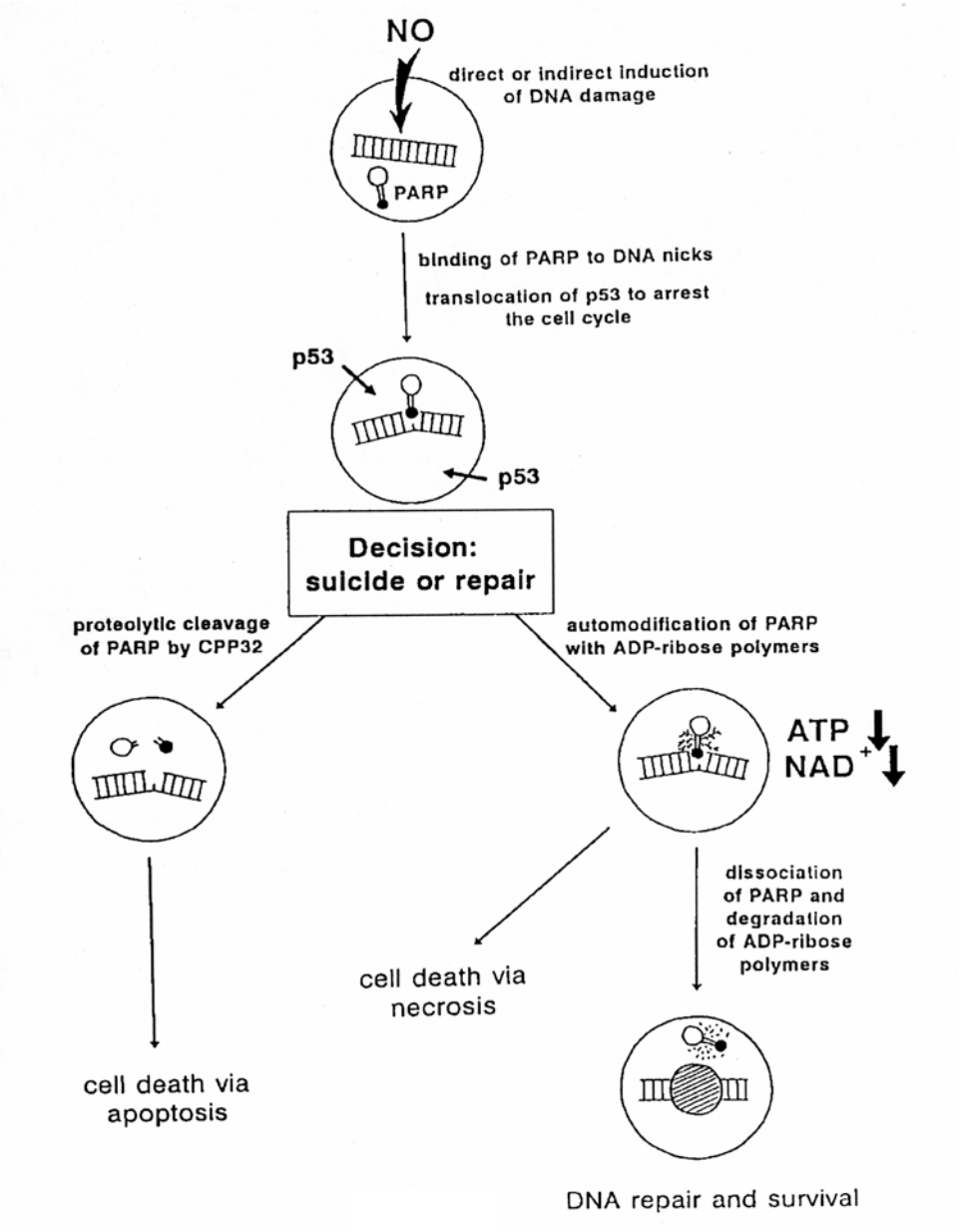

Fig 1.7 Effects of NO on nuclear DNA and response after DNA damage (Kroncke *et al.*, 1997).

1.2.3Effects of nitric oxide on some important cellular proteins

NO or its derivatives react with transition metals (iron, copper, zinc and so on) and thiol groups, therefore regulating protein functions and cell signaling pathways.

Reaction of NO with transition metals or metal-containing proteins is of high biological significance in NO-mediated cell signal transduction pathways. A classical example is the role of NO in cGMP-dependent signaling pathways (Cooper, 1999; Denninger and Marletta, 1999). NO binds to the ferrous heme of soluble guanylate cyclase and releases the heme-ligating histidine, resulting in a heme Fe^{2+}-NO complex. A change in the heme geometry then occurs causing a conformational change of the protein to an enzymatically active form. Active guanylate cyclase then results in an increase in the second messenger cyclic GMP (cGMP) and activates cGMP-dependent protein kinase (PKG) as well as phosphodiesterases, ion channels and other important regulatory proteins. This leads to: smooth muscle relaxation and blood pressure; platelet aggregation and disaggregation; neurotransmission both peripherally and centrally (Denninger and Marletta, 1999). NO also reversibly binds to ferrous heme iron of cytochromce *c* oxidase (discussed in 1.2.2) and inhibits its activity. Also, NO is able to bind reversibly to ferric iron, responsible for the inhibition of catalase (an enzyme for hydrogen peroxide conversion to water) by NO. Reaction of NO with the oxygen adduct of ferrous heme proteins (oxyhaemoglobin) is responsible for the metabolism in the vasculature. NO can also interact with iron-sulphur enzymes (aconitase, NADH dehydrogenase) (Cooper, 1999). Fig 1.8 summarize the most biologically relevant reactions between NO and iron.

NO^- is able to react with iron protein such as myoglobin, oxy- and deoxy-hemaglobin. $ONOO^-$ potently reacts with most of the iron proteins including myoglobin, hemoglobin and cytochrome *c* oxidase (Cooper, 1999).

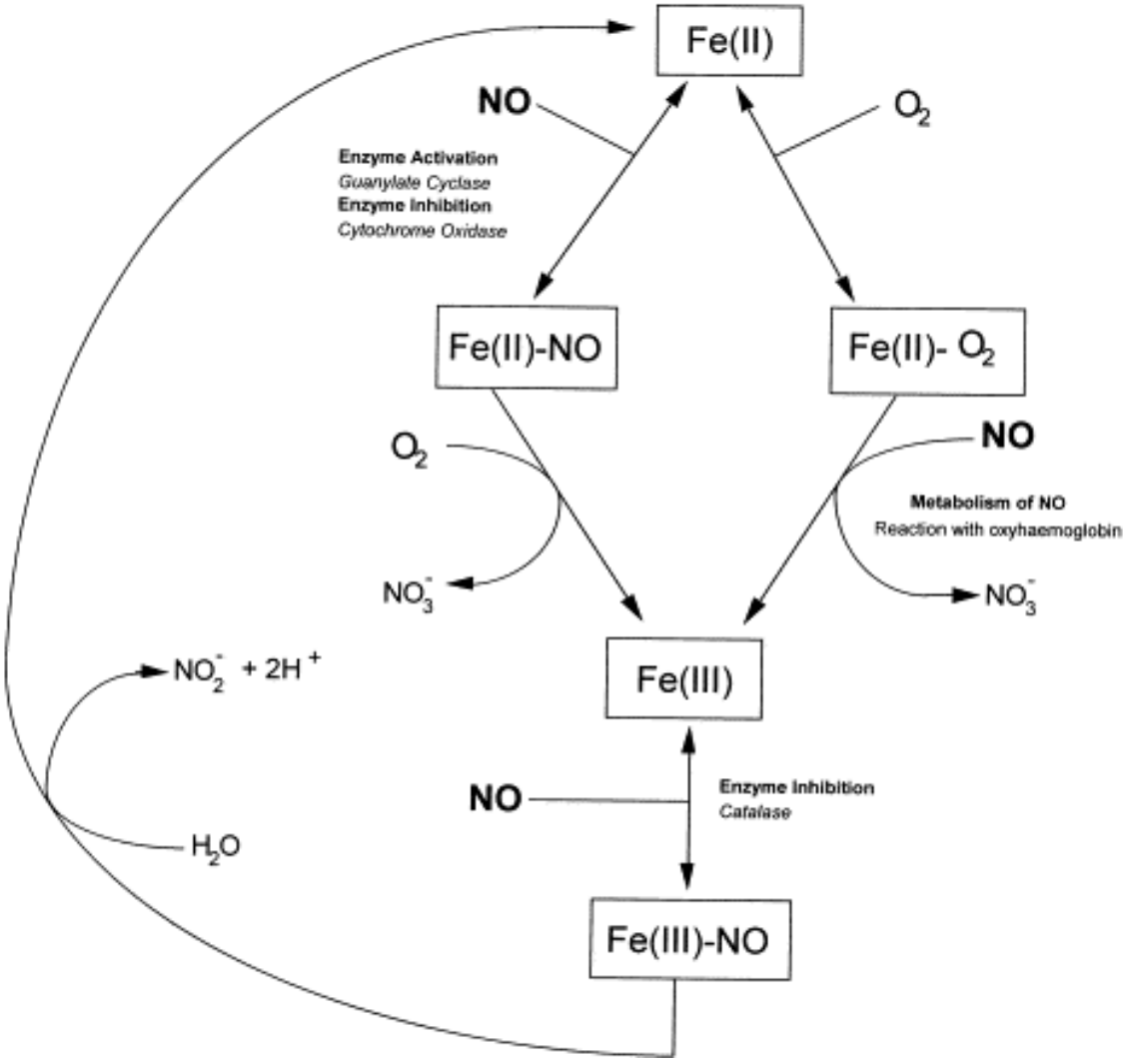

Fig 1.8 An overview of heme iron:NO interactions and their importance.

NO, especially NO^+ species, reversibly reacts with thiol groups (R-SH) as discussed in 1.2.1. It is now known that thiol-NO chemistry is relevant to immune, antimicrobial, smooth muscle relaxant and neuronal bioactivities (Gaston, 1999). The caspases are potential molecular targets of NO with respect to apoptosis. It was firstly

observed in cell-free *in vitro* studies that NO-related species dose-dependently inhibit recombinant caspases through *S*-nitrosation of an essential active site cysteine residue that is functionally conserved among these proteases (Dimmeler *et al*., 1997; Kim *et al*., 1998; Li *et al*., 1997). This inhibition was reversible and sensitive to reducing agents such as dithiothreitol (DTT) and glutathione (GSH). *S*-nitrosation was observed in almost all the 13 known mammalian caspases (Boyd and Cadenas, 2002). Two further studies established the physiological significance of this process. NO donors prevent apoptosis in HUVEC cells over-expressing caspase-3 by direct *S*-nitrosation of caspase-3 at the active site cysteine-163 (Rossig *et al*., 1999); Mannick *et al.* found that in human lymphocyte cell lines and MCF-7 cells, the inactive caspase-3 zymogen is also *S*-nitrosated at the same critical cysteine residue mediated by endogenously generated NO (Mannick *et al*., 1999). Fas-induced apoptosis in these cells involves the promotion of caspase activation by a dual process: de-nitrosation of the cysteine residue and cleavage of the zymogen to the active protease. The active caspase-3, in turn, could be further inhibited by *S*-nitrosation following an increase in the steady-state level of NO. So *S*-nitrosation of caspases may be a major mechanism of NO mediated cell protection against apoptosis (Brune *et al*., 1998; Lipton, 1999), inhibiting both the initiator and executor caspases and therefore preventing receptor-mediated as well as mitochondria-mediated apoptosis .

Besides the classical reaction of nitrosation (in which NO is transferred to –SH groups of a protein), NO is also able to enhance disulfide bonding of vicinal sufhydryl (thiol) groups of redox sensitive proteins. One example is the disulfide bonding in the redox sensitive modulatory site of the N-methyl-D-aspartate (NMDA) receptor complex, which is enchanced by NO nitrosation (Fig 1.9), thereby down-regulating its

Ca^{2+} channel activity and desensitizing the signaling pathway activated by NMDA (Landar and Darley-Usmar, 2003; Lipton, 1999).

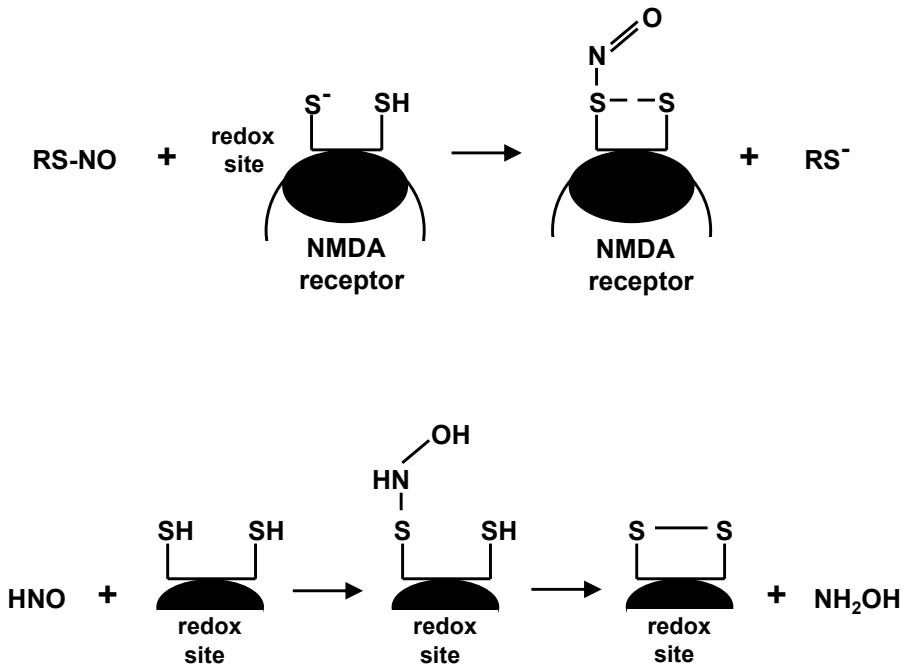

Fig 1.9 Schematic model of reaction of NO (in S-nitrosocysteine form, upper panel) and nitroxyl ion (NO^-, lower panel) with the NMDA receptor (Lipton, 1999).

The best example of NO activating a classical signaling pathway via *S*-nitrosation is the small guanine nucleotide binding protein H-Ras. *S*-nitrosation of a critical Cysteine (C118) residue causes guanine nucleotide (GDP/GTP) exchange that activates H-Ras. This results in the activation of a signaling pathway that includes ERK and NF-kappa B, and causes cellular proliferation and differentiation. The contribution of NO to the activation of Ras depends on a number of factors: the redox state of the cell (or subcellular region), the activation of the Ras pathway by other

effectors (like receptor tyrosine kinase) and the availability of competing targets of NO (Landar and Darley-Usmar, 2003).

1.2.4 Understanding the paradoxical effects of nitric oxide on cell viability

It is now clear that NO is able to exert either destructive or protective roles in different cell types. The consequence of NO exposure on cells depends on a number of poorly characterized factors. First of all, cell type plays an important role. Macrophages, thymocytes, neuronal cells, pancreatic islets, and some tumor cells are very sensitive to NO and undergo apoptosis or necrosis upon exposure to even low levels of NO. Other cell types (hepatocytes, human B lymphocytes, endothelial cells, cardiac myocytes, splenocytes, and ovarian follicles) are resistant to NO toxicity (Kim *et al*., 2000). Different cellular capacities to scavenge or to detoxify NO may be partially responsible for such variations in cell type susceptibility (Kroncke *et al*., 1997). The differences in the activity of the whole cellular antioxidant system, consisting of catalase, superoxide dismutases, glutathione reductase, glutathione peroxidase, thioredoxin, thioredoxin reductase to supply reduction equivalents, e.g., NADPH via the hexose monophosphate shunt, may lead to survival or cell death after nitrosative stress (Kroncke *et al*., 1997). Cell type-specific inducible defense mechanisms against NO may also account for the susceptibility difference. Hsp70, MnSOD, HO-1, Cox-2 upregulation have been reported in response to NO in different cell lines and neutralize the damaging effects of NO (Bogdan, 2001; Demple, 1999; Kroncke *et al*., 1997). Since the transactivation of a specific gene depends on the different cellular contexts, it is expected that different cells exhibit different patterns of these defense mechanisms that may account for the varying cellular susceptibilities.

The level of NO exposure and composition of NO-derived reactive species (RNS) are other key factors in determining the effects of NO on cell viability. For example, low concentrations of NO prevent apoptosis in serum-starved PC12 cells while high concentrations lead to necrotic cell death (Kim *et al*., 1999). One of the mechanisms underlying apoptosis resistance is caspase nitrosation by NO (discussed in 1.2.3), which inhibits both initiator (e.g. caspase-8) and executor caspases (e.g. caspase-3) so that the cytochrome *c* induced initiating cell death pathways and the positive feedback amplification of apoptotic signaling arising from the downstream promotion of cytochrome *c* release by pre-activated caspase-3 are both greatly prevented (Boyd and Cadenas, 2002). It is worth pointing out that *S*-nitrosation is not exclusive and alternative mechanisms might also be important, depending on the cell-type and nature of the pro-apoptotic signals. That may partially explain why NO can inhibit caspases in some scenarios while activate caspases in the others. At high concentration of NO, the electron flux through mitochondrial respiratory chain is severely damaged (discussed in 1.2.1 and 1.2.2), excessive $O_2^{\bullet -}$ is generated and thus production of $ONOO^-$ is highly favored. Peroxynitrite further causes irreversible damage to mitochondria ATP synthase and aconitase, and promotes the permeability transition, cytochrome *c* release and apoptosis. Fig 1.10 briefly describes how the dual roles of NO in cell death are achieved.

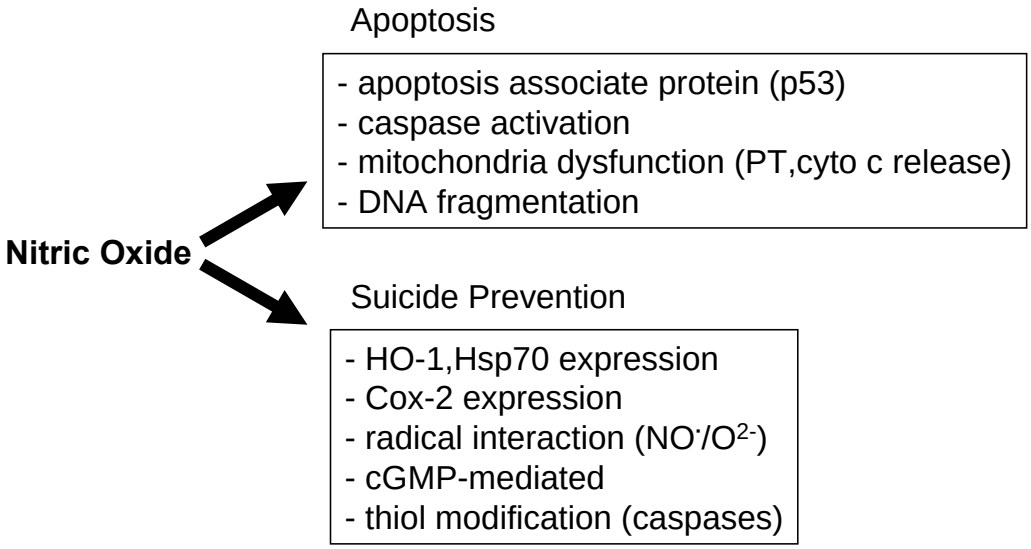

Fig 1.10 The dual roles of NO on cell viability and the possible explainations (Brune *et al.*, 1998).

1.3 AP-1 and programmed cell death

1.3.1 Regulation of AP-1 activity as a transcription factor

AP-1 (activator protein 1) is a homo- or hetero-dimer consisting of basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2) and the closely related activating transcription factors (ATF2, LRF1/ATF3, B-ATF) subfamilies (Karin *et al*., 1997). These protein families contain three important domains: a transactivation domain (TAD), a DNA binding domain (DBD) and a leucine zipper region. The Jun proteins are most versatile and capable of forming Jun-Jun, Jun-Fos and Jun-ATF dimers. The dimerization occurs via hydrophobic interactions between the leucine zipper regions. Jun-Jun and Jun-Fos dimers bind TREs (TPA-responsive elements, 5'-TGAG/CTCA-3'), while Jun-ATF dimers prefer CREs (cAMP responsive elements, 5'-TGACGTCA-3') (Hai and Curran, 1991). A number of other bZIP proteins which can heterodimerize with Jun, Fos and ATFs have also been characterized (see review by Karin *et al*., 1997).

AP-1 activity is induced by a broad range of extracellular stimuli including growth factors, hormones, cytokines, cell-matrix interactions, bacterial or viral infections and genotoxic agents (Shaulian and Karin, 2002). In general, the regulation of AP-1 activity occurs through: first, changes in gene transcription and mRNA turnover; second, effects on protein turnover; third, post-translational modification that modulate the transactivation potential; and fourth, interactions with other transcription factors that can either synergize or interfere with AP-1 activity. In the following paragraphs, regulation of c-Jun, c-Fos and ATF2 activity will be discussed as a paradigm.

1.3.1.1Transcriptional regulation of *c-jun* and *c-fos* expression

Most of the genes encoding AP-1 components belong to "immediate-early" genes whose transcription is rapidly induced, independently of *de novo* protein systhesis following cell stimulation. Among them, the regulation of *c-fos* and *c-jun* transcription is best understood (Karin, 1995).

c-jun is expressed in various cell types at low levels, and its expression is elevated in response to many stimuli. Induction is usually mediated through the *c-jun* TRE, which was recognized by c-Jun·ATF2 heterodimers (van Dam *et al*., 1993). Exposure of cells to UV irradiation, proinflammatory cytokines or growth factors results in activation of the JNK and p38 groups of MAPKs. JNKs and p38 can further phosphorylate c-Jun and ATF2, thus stimulating their transcriptional activity (Fig 1.10) (Karin, 1995).

Compared with *c-jun*, the *c-fos* promoter region is more complicated. *c-fos* transcription can be stimulated very rapidly and transiently, partly due to the existence of several *cis* elements mediating *c-fos* induction. Proximal to the *c-fos* TATA box is a CRE that is potentially occupied by CREB (CRE-binding protein) or ATF proteins, which all mediate *c-fos* induction via cAMP- and Ca^{2+}-dependent signaling pathways in response to neurotransmitters and hormones (Karin *et al*., 1997). The phenotypic similarities of *c-fos*$^{-/-}$ and *ATF2*$^{-/-}$ mice (Johnson *et al*., 1992; Reimold *et al*., 1996), both of which have defects in bone formation and the central nervous system, suggest that ATF2 may regulate *c-fos* expression in these tissues. However, the *CREB*$^{-/-}$ and *CREM*$^{-/-}$ mice displayed different phenotypes from *c-fos* and *ATF2* null mice (Blendy *et al*., 1996; Hummler *et al*., 1994), suggesting that neither CREB nor CREM is critical in regulation of *c-fos* in vivo. Another *cis* element that regulates *c-fos* transcription is a

Sis-inducible enhancer (SIE) which is recognized by transcription factors of the STAT (signal transducer and activator of transcription) family which are activated by the JAK group of kinases. A third *cis* element is the serum-response element (SRE), which is recognized by a dimer of the serum-response factor (SRF) that recruits the ternary complex factors (TCFs). TCFs are important mediators of *c-fos* induction by a large variety of extracellular stimuli. For example, mitogen-induced *c-fos* transcription is largely dependent on ERK-mediated Elk (one component of the TCFs) phosphorylation and increase of its transcriptional activity; UV irradiation and IL-1-induced *c-fos* transcription is through JNKs or p38-mediated Elk phosphorylation (Fig 1.11) (Karin, 1995; Karin *et al*., 1997).

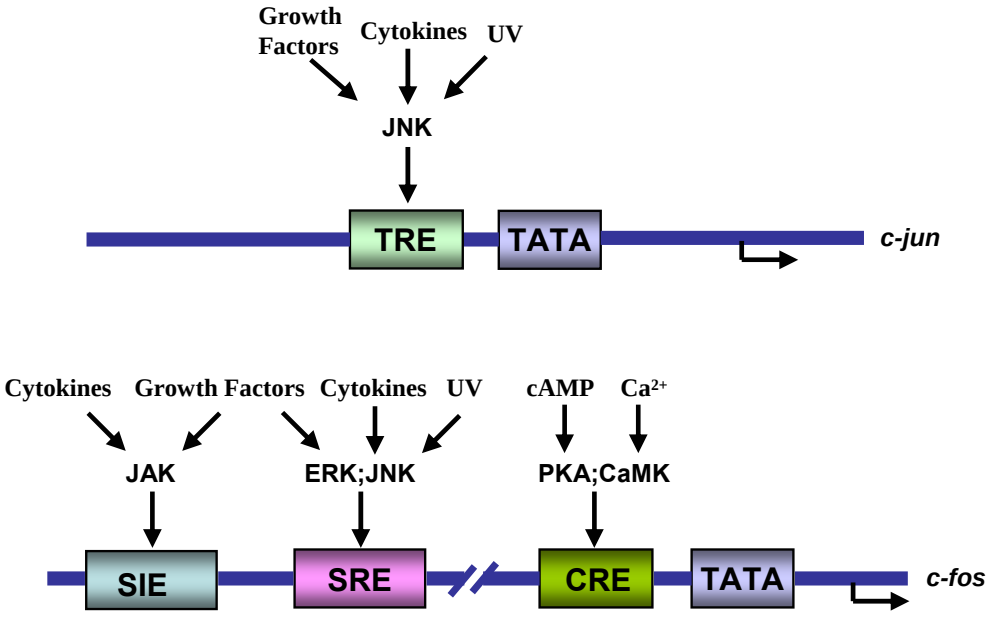

Fig 1.11 Regulation of *c-jun* and *c-fos* transcription. The cis-elements mediating *c-jun* and *c-fos* transcription in response to different stimuli are illustrated. The protein kinases that phosphorylate the transcription factors that interact with these elements are indicated. *PKA*, protein kinase A; *CaMK*, calmodulin-dependent kinase; *SIE*, Sis-inducible enhancer; *CRE*, cAMP response element (Karin, 1995).

Unlike c-Jun and c-Fos, ATF2 is a constitutively expressed. Activity regulation of ATF2 mainly occurs at the posttranslational level.

1.3.1.2 Posttranslational regulation of c-Jun, c-Fos and ATF2

The activities of AP-1 components (preexisting and newly-synthesized) are regulated by phosphorylation. This form of posttranslational modulation is best illustrated in c-Jun, c-Fos and ATF2. It is possible that other proteins in this family are also subject to similar regulation.

The activity of c-Jun is primarily regulated by the JNK (c-Jun N-terminal kinase) family of MAP kinases by specific phosphorylation of serine 63 and 73 of c-Jun within its transactivation domain, resulting in a large increase in the ability of c-Jun to interact with the CBP/p300 family of transcription coactivators and to cause transcriptional activation (Karin, 1995). c-Jun is also phosphorylated by ERK or GSK-3 at a cluster of sites located next to its basic region which can inhibit its DNA binding activity (Boyle *et al*., 1991; Chou *et al*., 1992; Minden *et al*., 1994).

The sequence surrounding the N-terminal phospho-sites of c-Jun is conserved in the C-terminal activation domain of c-Fos, suggesting a similar mode of regulation. Thr-232, the homolog of Ser-73 of c-Jun, is phosphorylated by FRK (Deng and Karin, 1994) and potentiates c-Fos transcriptional activity. The mechanism by which phosphorylation of Thr-232 enhances c-Fos transcriptional activity is less clear, perhaps through interaction with components in the transcription machinery as in the case of phosphorylation of Ser-63 and Ser-73 of c-Jun.

ATF2 phosphorylation at Thr-63 and Thr-71 within its N-terminal transactivation domain is also proven to stimulate its transcriptional activity. Like c-Jun, ATF2 is also phosphorylated by the JNKs. Also, transactivation by ATF2 is also potentiated upon

binding of Rb or E1A, probably through recruitment of additional activation domains to the DNA-bound ATF2 dimers (Karin, 1995).

1.3.1.3 Regulation of AP-1 activity by its interacting proteins

Important AP-1 interacting proteins which may regulate its transcriptional activity are three different types of MAPKs (mitogen activated protein kinases) as discussed in 1.3.1.1 and 1.3.1.2, including JNKs, ERKs and p38s. These MAPKs are highly specific in selecting their substrates in stimuli-dependent fashions (Karin, 1995).

Another protein that regulates AP-1 activity is a transcriptional coactivator called JAB1 (Jun activation domain binding protein 1). JAB1 efficiently interacts with c-Jun and JunD, irrespective of phosphorylation of c-Jun, but not with JunB (Claret *et al*., 1996). The mechanism by which JAB1 activates c-Jun and JunD is through stabilization of the complexes that these proteins form with the AP-1 sites. The selective interaction of JAB1 with c-Jun and JunD, but not with JunB, might account for the differential target gene activation by these proteins (Karin *et al*., 1997). Target gene specificity is also determined by other AP-1 interacting protein. In the case of the Jun-Fos dimer, it has been shown that interaction between the dimer and other bZIP proteins (Maf, NF-E2), non-bZIP DNA-binding proteins (NFAT, Smad) contributes to the functional specificity of Jun-Fos proteins (Chinenov and Kerppola, 2001).

Besides positive regulation of AP-1 activity, negative regulatory mechanisms also exist. For example, TPA-induced AP-1 activity is inhibited by the interferon-inducible p202 through inhibition of DNA-binding of c-Jun:c-Jun or c-Jun:c-Fos dimmers (Min *et al*., 1996). Another protein established to suppress AP-1 activity is JDP2 (Jun-dimerizing partners 2). JDP2 is associated with c-Jun in the cell and strongly inhibits c-Jun transcriptional activity (Aronheim *et al*., 1997).

1.3.2 Role of AP-1 in cell proliferation and differentiation

1.3.2.1 Role of AP-1 in cell proliferation

Initially AP-1 was found to be a mediator of oncogenic transformation based on the following observations: AP-1 activity is induced by various oncogenic signals, including TPA, growth factors and oncogenic viruses. Transformation by activated Ras, Raf or MEK1 induces AP-1 protein expression (Jochum *et al*., 2001). The transforming activity of AP-1 suggests that it is involved in either stimulation of cell proliferation or suppression of cell death. Some of the key experimental data will be described implicating AP-1 proteins in growth control and transformation.

1.3.2.1.1 AP-1 is an important regulator in cell proliferation

Requirement of c-*fos* and c-*jun* for proliferation as well as cell cycle progression was demonstrated through inhibition of their expression using antisense RNA. Consistently, microinjection of antibodies against Fos and Jun prevented serum-stimulated cell cycle re-entry in quiescent mouse fibroblasts (Shaulian and Karin, 2001; Shaulian and Karin, 2002). Studies using fibroblasts derived from *fos* and *jun* null mice partially supported the observations above. *c-fos*$^{-/-}$*fosB*$^{-/-}$ fibroblasts showed reduced proliferation capacity while *c-fos*$^{-/-}$ or *fosB*$^{-/-}$ fibroblasts proliferated normally; moreover, *c-fos*$^{-/-}$*fosB*$^{-/-}$ mice were smaller than their wild type counterparts while the single knockout mice developed normally (Brown *et al*., 1998; Brusselbach *et al*., 1995). Similarly, a reduced fibroblast proliferation rate as well as smaller cell size were also characterized in *junD*$^{-/-}$ mice (Thepot *et al*., 2000). Among all the different AP-1 deficiencies, mouse embryo fibroblasts (MEFs) from *c-jun*$^{-/-}$ embryos bore the most severe proliferation defects (Johnson *et al*., 1993; Schreiber *et al*., 1999). The cells can only be passed in culture once or twice before entering premature senescence.

Furthermore, even after immortalization, they still proliferated at a much slower rate than the wild type cells (Shaulian and Karin, 2002). Although JNK-mediated phosphorylation of c-Jun within the transactivation domain dramatically enhances c-Jun activity, it is only partially responsible for stimulation of cell proliferation, as *c-jun*$^{Ala63/73}$ fibroblasts have a proliferation defect less severe than that of *c-jun*$^{-/-}$ fibroblasts (Behrens *et al*., 1999). Besides the role in continuous cell proliferation, c-Jun induction also seems required for cell cycle re-entry in UV-irradiated fibroblasts (Shaulian *et al*., 2000). While the wild type fibroblasts only undergo transient cell cycle arrest after UV exposure, *c-jun*$^{-/-}$ cells experience prolonged growth arrest and fail to resume proliferation. c-Jun is also important for proliferation in other cell types such as fetal hepatoblasts and contributes to liver regeneration. Collectively, AP-1 activity is essential for cell proliferation.

However, not all AP-1 proteins support cell proliferation. JunB, a Jun subfamily protein, has been shown to negatively regulate cell proliferation and antagonize c-Jun activity. Examples are: *junB*$^{-/-}$ MEFs proliferate normally, similar to the wild type cells; MEFs from *junB* transgenic mice showed limited proliferation capacity and a prolonged G1 phase when immortalized in culture; *c-Jun*$^{-/-}$ fibroblasts are deficient in keratinocyte proliferation while *JunB*$^{-/-}$ fibroblasts proliferate faster than wild type cells (Shaulian and Karin, 2002).

The effects of JunD on cell proliferation are hard to understand. The *junD*$^{-/-}$ MEFs are defective in proliferation and display premature senescence partially due to increased expression of *p19*^{ARF}, a tumor suppressor gene that enhances p53 activity. In contrast, the immortalized *junD*$^{-/-}$ cells show an enhanced proliferation rate, probably arising from overexpression of c-Jun as a compensating mechanism and consequent *cyclin D1* upregulation (Shaulian and Karin, 2002).

1.3.2.1.2 Mechanisms of AP-1 modulation of cell proliferation

AP-1 proteins were shown to bind directly and activate the *cyclin D1* promoter in transient transfections (Shaulian and Karin, 2001). This observation provides a possible connection between AP-1 family proteins and the cell cycle machinery. Indeed, the induction of cyclin D1 by serum is more efficient in wild type fibroblasts than in *c-fos*$^{-/-}$*fosB*$^{-/-}$ double mutants (Brown *et al*., 1998). Consistently, reduced expression of cyclin D1 is also observed in *c-jun*$^{-/-}$ MEFs (Wisdom *et al*., 1999). However, this deficiency does not fully account for the apparent proliferation defects in the cells since overexpression of cyclin D1 can only partially restore the capacity of proliferation in the *c-jun* null MEFs. Instead, the defect of proliferation is able to be completely suppressed by simultaneous loss of p53 protein (Shaulian and Karin, 2002). Moreover, c-Jun acts as a direct suppressor of *p53* transcription as well as inhibitor of p53 activity, resulting in a failure to activate p21^{Cip1} (a CDK inhibitor). As a consequence, p53 is accumulated in *c-jun*$^{-/-}$ cells through enhanced transcription of the *p53* gene, leading to *p21* expression and cell cycle arrest. Thus, c-Jun can stimulate cell cycle progression through two distinct mechanisms: direct induction of *cyclin D1* transcription and indirect repression of *p21* transcription (Shaulian and Karin, 2002).

As a c-Jun antagonist, JunB represses the *cyclin D1* promoter (Bakiri *et al*., 2000) and furthermore, induces the transcription of *p16*^{INK4a} (Passegue and Wagner, 2000), coding for a CDK inhibitor that inhibits Rb phosphorylation by CDKs and therefore G1 to S phase transition (Fig1.12).

1.3.2.1.3 AP-1 is a mediator of oncogenic transformation: the mechasnisms

AP-1 proteins are actively involved in controlling cell proliferation through modulation of the cell cycle regulators, suggesting they could be potentially involved in transformation.

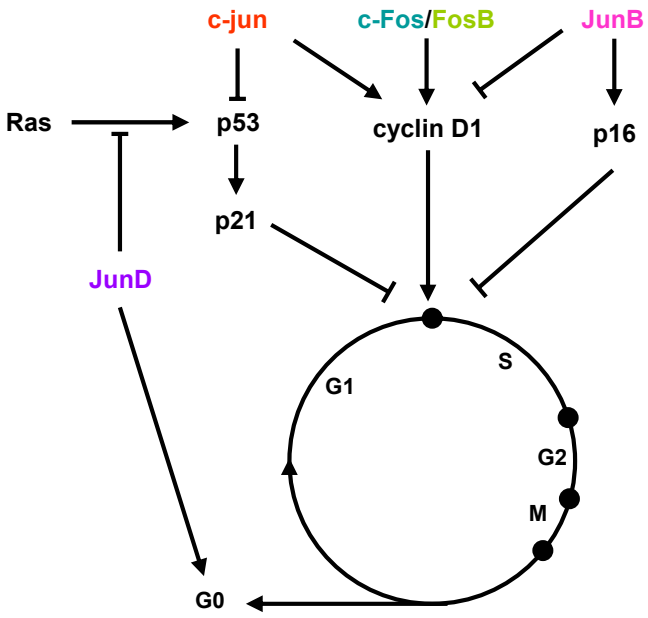

Fig 1.12 Effects of AP-1 proteins on cell cycle regulation. AP-1 proteins control cell cycle progression by regulating the expression of key components of the cell cycle machinery. C-jun stimulates G1 to S phase transition by inducing cyclin D1 and repressing p53, which in turn reduces p21 levels. C-Fos and FosB have redundant functions in the stimulation of S phase entry and the induction of cyclin D1 expression. JunB inhibits G1 to S phase progression by inducing p16 and repressing cyclin D1. JunD inhibits S phase entry and increases the numbers of resting cells by modulating the Ras/p53 pathways (Jochum *et al*., 2001).

When expressed *in vivo*, c-Fos transforms chondroblasts and osteoblasts, contributing to c-Fos-induced tumorigenesis of the skeleton (Jochum *et al*., 2001). The oncogenic activity of c-Fos is dependent on its dimerization with Jun proteins as well

as DNA binding. The integrity of the structural motifs within its N- and C-terminal transactivation domains is also essential. c-Fos–induced transformation is partly mediated by induction of the enzyme DNA methyl transferase 1 (DNMT1), which downregulates the expression of negative growth regulators through methylation of their promoter region (Shaulian and Karin, 2002). FosB transforms fibroblasts in culture but this does not happen *in vivo*. Fra-1 and Fra-2 have weaker transforming activity compared to other Fos proteins (Jochum *et al*., 2001).

c-Jun is able to cause oncogenic transformation in mammalian cells, but relies on coexpression with an activated oncogene such as Ras or Src (Schutte *et al*., 1989). Transformation by c-Jun requires the intact N-terminal transactivation domain and can be enhanced by c-Jun phosphorylation on Ser-63 and Ser-73 (Jochum *et al*., 2001), implicating c-Jun target genes as mediators of transformation. Several genes involved in the transforming activity of Jun proteins in CEFs (chicken embryonic fibroblasts) have been identified (Fu *et al*., 1999; Hartl *et al*., 2001) including *JAC* (Jun-activated gene in CEF) and *HB-EGF* (heparin-binding epidermal growth factor-like growth factor). In addition, different dimerization partners of c-Jun have important influences on its transformation-related activities (Shaulian and Karin, 2002): the c-Jun:c-Fos dimer is efficient in transforming skeletal osteoblasts and induces anchorage-independent growth; and the c-Jun:ATF2 dimer promotes growth factor-independent growth. However, the differentially regulated target genes in these circumstances remained to be identified. Despite its oncogenic potential *in vitro*, c-Jun overexpression in transgenic mice does not lead to tumorigenesis (Grigoriadis *et al*., 1993). Unlike c-Jun, both JunB and JunD lack transforming activity and may instead act as anti-oncogenes. This is supported by the observation that *junB*$^{-/-}$ mice are susceptible to developing a disease resembling human chronic myeloid leukemia

(Passegue *et al*., 2001). This sensitivity arises from the increased levels of the GM-CSFα receptor and of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, as well as decreased levels of the cell cycle inhibitor p16, suggesting that JunB inhibits proliferation and thus suppresses leukaemogenesis (Jochum *et al*., 2001).

1.3.2.2 Role of AP-1 in cell differentiation

Differentiation is an important process for multicellular organisms to generate functionally discrete organs/tissues. Temporal changes in gene expression that result in cellular biochemical alterations and growth arrest are primary requisites for terminal differentiation. The modulation of gene expression is associated with the activities of transcription factors which promote or inhibit gene transcription (Kang *et al*., 1998). Multiple roles of AP-1 proteins in differentiation are well characterized in the hematopoietic lineages (see review by Liebermann *et al*., 1998).

Hematopoiesis is a process in which a hierarchy of hematopoietic progenitor cells in the bone marrow (BM) proliferate and terminally differentiate along multiple distinct cell lineages. During the process, cell proliferation, differentiation and apoptosis are well-balanced to reach homeostasis. It has been proposed that coordinated expression of lineage specific and cell specific transcription factors plays pivotal roles in converting the proliferative and differentiative signals into genetic programs which determine cell survival, growth, differentiation, and functional activation as well as cell death (Liebermann *et al*., 1998). Fig 1.13 summarizes the key transcription factors involved in these processes.

In vitro studies indicate that AP-1 complexes are functionally involved in regulation of hematopoietic progenitor development along most of the myeloid lineages including the monocyte/macrophage, granulocyte, megakaryocyte and erythroid lineages. The evidence is the murine M1 myeloid leukemic cell line can be

differentiated into macrophages upon stimulation by IL-6 and LIF (leukemia inhibitory factor), during which *c-jun*, *junB*, *junD* were stably induced (Lord *et al*., 1993). Overexpression of *c-fos* in M1 cells results in a marked increase in the cell population capable of being induced to differentiate at much lower (50- to 100- fold) concentrations of IL-6 compared with the wild type cells; *IL-6*, a differentiation inducer of M1 cells, is also an AP-1 target gene (Ray *et al*., 1989) during differentiation; *c-fos* antisense oligonucleotides dramatically impaired the differentiation capacity of M1fos cells at low concentrations of IL-6 and also inhibited the ability of normal BM-derived myeloblasts to differentiate into mature macrophages or granulocytes induced by M-CSF (macrophage colony stimulating factor) and G-CSF (granulocyte colony stimulating factor) (Lord *et al*., 1993).

In vivo studies using *c-fos* null mice helped to establish a unique role of c-Fos in determining the differentiation and activity of progenitors of the osteoclast lineage (Johnson *et al*., 1992; Wang *et al*., 1992), a specific population of bone-forming cells which are of hematopoietic origin. *c-fos*$^{-/-}$ mice develop a bone disease osteopetrosis, a disorder characterized by defective osteoclast function and increase in skeletal mass, due to a complete block of osteoclastic progenitor cells to differentiate into mature functional osteoclasts. This defect is only limited to osteoclastic progenitor cells and maturation of cells of the osteoblast/chondrocyte lineages (multipotent mesenchymal progenitor cells origin) is not affected.

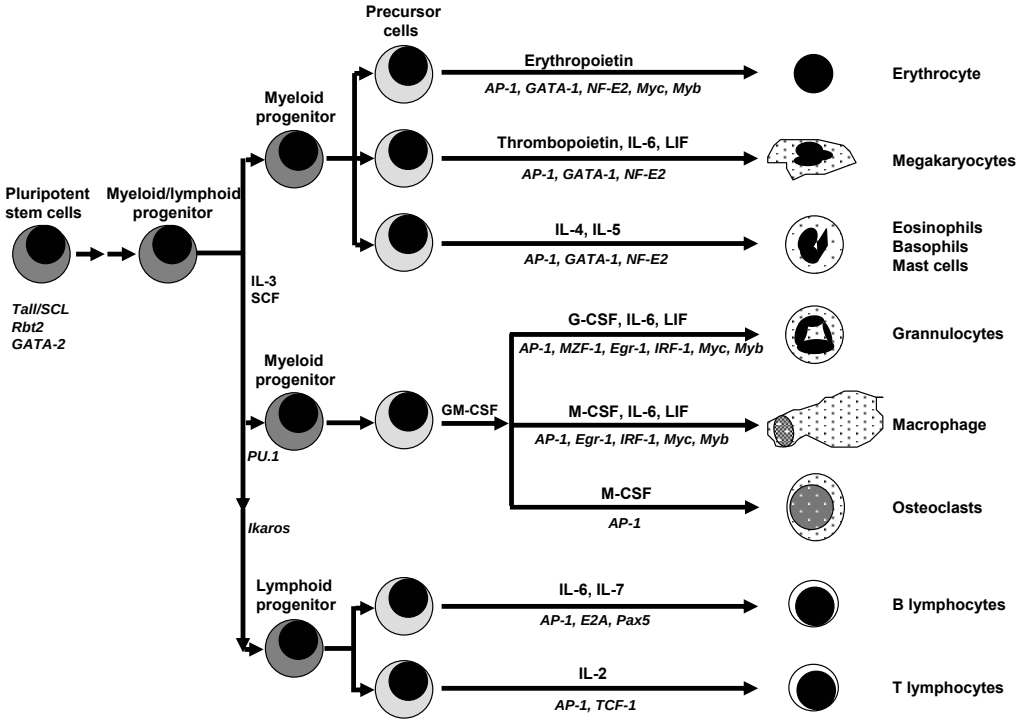

Fig 1.13 Hematopoietic lineages and transcription factors as well as the major hematokines essential for their development. IL, interleukin; CSF, colony stimulating factor; G-CSF, granulocyte CSF; GM-CSF, granulocyte-macrophage CSF; M-CSF, macrophage CSF; LIF, leukemia inhibitory factor (Liebermann *et al*., 1998).

AP-1 proteins also modulate T- and B-cell development. T cell activation is responsive to IL-2. CREB undergoes rapid phosphorylation on Ser119 upon IL-2 stimulation and facilitates AP-1 activation and subsequent IL-2 production, which drives T cells into cell cycle progression (Barton *et al*., 1996; Jain *et al*., 1994). In addition, c-Fos apparently plays a negative role in regulating B-cell development. Evidence came from the analysis of transgenic mice overexpressing *c-fos*. The

proliferation and development of B lineage cells is retarded in BM cell cultures obtained from transgenic mice constitutively expressing *c-fos* and blocked in BM cells obtained from transgenic mice expressing IFN (interferon)-inducible *c-fos*, upon stimulation with IFN (Liebermann *et al*., 1998).

Besides, AP-1 proteins have been shown to enhance differentiation in melanoma and thus prevent the abnormal growth of melanoma cells (Kang *et al*., 1998), pointing out a possible target in tumor therapeutics.

1.3.3 Role of AP-1 in cell death

Although initial studies suggest a role for AP-1 proteins in promoting cell survival or suppressing cell death, there is evidence that AP-1 is implicated in triggering cell death. The earliest indication of apoptotic role of AP-1 came from the following observations: lasting induction of c-Fos in the brains of mice treated with kainic acid, an activator of glutamate receptor and inducer of hippocampal neurons apoptosis (Smeyne *et al*., 1993); robust induction of c-Jun before the onset of cell death after exposure of the cells to various genotoxic stresses such as UV irradiation (Devary *et al*., 1991) or alkylating agents.

Direct proof that AP-1 is functionally involved in apoptosis came from the analyses of animals or cells lacking AP-1 expression or activity. It is now clear that AP-1 functions in apoptosis in several different scenarios: normal development and terminal differentiation to remove unwanted cells and maintain homeostasis; and pathology resulting from acute or chronic disorders. The role of AP-1 in these cases will be discussed in detail in the following paragraphs.

Using *fos-LacZ* transgenic mice, Smeyme *et al* found a continuous high expression of *fos-LacZ* in skin, hair follicles and bone cells undergoing terminal

differentiation and culminating in apoptotic cell death (Smeyne *et al*., 1993). Also, in the ovary, *fos-lacZ*-positive follicular cells were associated with atreic follicles/corpora lutea that are degenerating while oocyte containing follicles were negative for *fos-LacZ* expression. Although indirect, these data suggested that AP-1 plays a general role in apoptosis during early development and postnatally, upon terminal differentiation of many cell types. Another example of AP-1 involvement in development-related apoptosis is in the neuronal system. About half of the original population of neurons is eliminated by apoptosis during the development of the nervous system (Ameyar *et al*., 2003). This kind of cell death is critical for ensuring the correct connections in the brain and partly due to competition of neurons for limited amounts of trophic factors such as NGF. NGF deprivation causes a rapid JNK activation as well as c-Jun phosphorylation (Le Niculescu *et al*., 1999; Watson *et al*., 1998). Conditional knockout of c-Jun in sympathetic neurons enhanced the survival of the neurons, proving the essential role of c-Jun in NGF deprivation-induced apoptosis in neurons (Palmada *et al*., 2002).

Two well-documented examples of AP-1 involvement in enhancing apoptosis arising from pathology are: in neurons, upregulation of *Bim* or other BH3-only Bcl-2 family members such as *DP5/Hrk*, in JNK/c-Jun dependent style may represent general mechanisms underlying neuronal apoptosis (Harris and Johnson, Jr., 2001; Lei and Davis, 2003; Whitfield *et al*., 2001). The obvious resistance of *jnk3*$^{-/-}$ and JunAA mice to epileptic seizures and neuronal apoptosis in the hippocampal region induced by kainic acid (Behrens *et al*., 1999; Yang *et al*., 1997) might be partially due to blockade of these pathways. More importantly, it was recently demonstrated that the neurotoxic β–amyloid peptide (Aβ), an important protein in the pathogenesis of Alzheimer's disease, induced apoptosis of cerebral endothethlial cells via AP-1

activation and subsequent Bim induction (Yin *et al*., 2002). In lymphoid cells, JNK-c-Jun-*FasL* is responsible for both the proper action of the immune system (in T-cell mediated cytotoxicity) and immune cell homeostasis (during the deletion of peripheral lymphocytes after the immune response) (Ameyar *et al*., 2003). It is worth pointing out that activation of the JNK-c-Jun-*FasL* pathway also results in apoptosis in other cell types such as neuronal cells or fibroblasts (Kolbus *et al*., 2000; Le Niculescu *et al*., 1999), which respond to extrinsic cell death pathways.

The function of AP-1 in the cellular response to genotoxic stress is less conclusive. There are several studies suggesting a proapoptotic role of AP-1 in UV-induced cell death. *jnk1*$^{-/-}$*jnk2*$^{-/-}$ MEFs are relatively resistant to UV (Tournier *et al*., 2000); *c-Jun*$^{-/-}$ fibroblasts are resistant to alkylating agents as well as UV(Kolbus *et al*., 2000b; Shaulian *et al*., 2000); dominant negative c-Jun reduces apoptosis in human monoblastic leukemia cells exposed to a variety of genotoxins (Verheij *et al*., 1996). However, other studies suggest a protective role of AP-1 against UV (Ivanov *et al*., 2001; Wisdom *et al*., 1999). This may be mediated through cooperation with STAT3 to suppress the transcription of *fas*.

A clear example of the anti-apoptotic activity of AP-1 is in liver cells, since *c-jun*$^{-/-}$ cells die from defects in hepatogenesis (Hilberg *et al*., 1993). AP-1 is suggested to suppress p53 thus enhancing cell survival through multiple mechanisms including modulation of p19^{Arf} (a protein that stabilizes p53), direct inhibition of *p53* transcription or p53 activity as a transcription factor in DNA binding or substrate recognition specificity (see review by Ameyar *et al*., 2003). Furthermore, some of the protective effects of AP-1 might be mediated through induction of *bcl-3*, whose product functions as a survival factor for growth factor-deprived T cells (Rebollo *et al*., 2000).

Two models were proposed to explain the diverse effects of AP-1 on cell death and survival (Ameyar *et al*., 2003). Model 1: induction of AP-1 results in activation of various target genes, such as *fasL*, *bim*, or *bcl-3* whose products either promote or suppress cell death. The balance between pro- and anti-apoptotic genes finally determines whether the cell dies or survives (Fig 1.14A). This balance may vary from one cell type to another, and may be dependent on the type and duration of stimulus used to activate AP-1, as well as on the activation of other transcription factors. This would help to explain the complicated and even paradoxical role of AP-1 in different conditions. Model 2: AP-1 functions as a homeostatic regulator that keeps cells in a certain proliferative steady state. Changes in the environmental conditions may enhance AP-1 activity. Persistent activation of AP-1 in cells containing damaged DNA causes defective replication and may trigger apoptosis through the same mechanisms that induce cell death after constitutive expression of oncogenes. However, the activation of AP-1 in cells that are able to proliferate promotes cell proliferation and survival (Fig 1.14B).

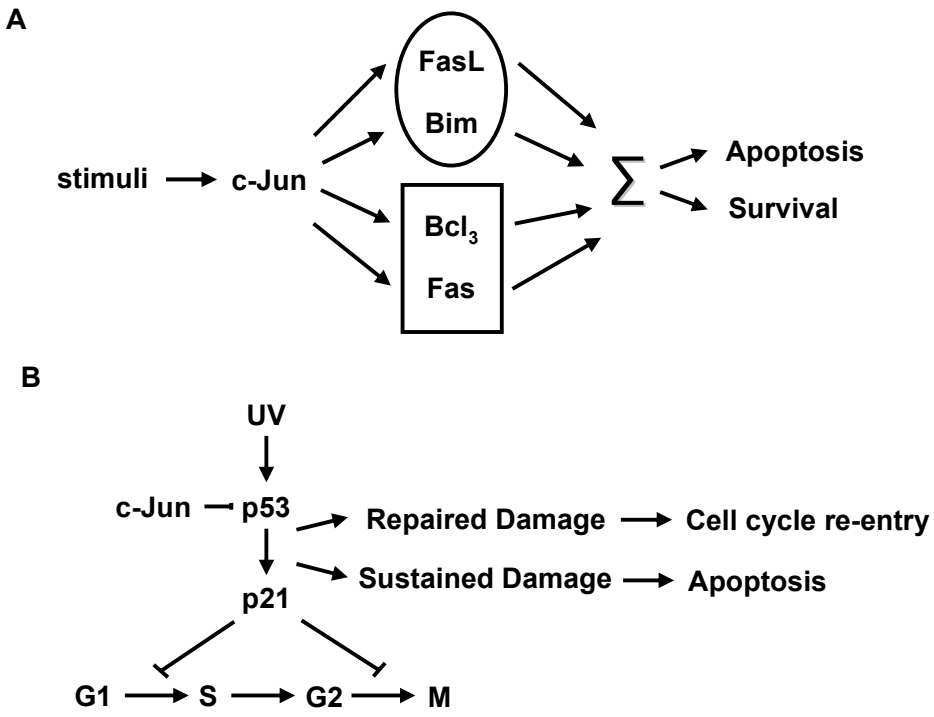

Fig 1.14 Effects of c-Jun on apoptosis. (A) Direct effects of c-Jun on apoptosis through transcriptional regulation. The sum of all signals (pro- and anti-apoptotic gene products) determines the cell fate. (B) Indirect effects of c-Jun on apoptosis. C-Jun activation results in downregulation of p21 expression and thus allows the cell cycle to progress. However, the actual fate of cells will be determined by the extent of cell damage (Shaulian and Karin, 2002).

1.4 Thesis rationale

Although the AP-1 family of transcription factors was one of the first identified in mammals, its physiological functions are still far from being elucidated, especially in terms of AP-1 function in cell death and survival. Under some circumstances, AP-1 activation results in cell death while in others, activation of AP-1 protects the cell from death. AP-1 activation has also been shown to have nothing to do with cell death or survival. Even more complex, AP-1 could have different or opposite functions in different types of cells in response to one single stimulus. It is proposed that the diverse effects of AP-1 on cell death and survival arise from activation of different group of target genes with pro- or anti-apoptotic properties and the balance between them determines the final outcome. The nature and duration of the stimuli, tissue origin and cell specificity are main determinants for the activation. However, identification of pathway-specific AP-1 target genes is still at an early stage. In our laboratory, the human neuroblastoma cell lines (SH-Sy5y and SHEP) are used as an *in vitro* model of neuronal cells to investigate the possible roles of AP-1 in response to NO. The main purposes of my research work are:

1). To investigate the signal transduction pathways that mediate AP-1 activation in response to nitric oxide (NO) in neuroblastoma cells. Since AP-1 activity is posttranslationally regulated by MAPK, I focused on the study of MAPK;

2). To identify the NO-inducible, AP-1-dependent target genes which mediate the function of AP-1 in response to NO in neuroblastoma cells.

CHAPTER 2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Bovine serum albumin (BSA), ampcilin, kanamycin, penicillin-streptomycin, SNP, SIN-1, DETA-NO, pyruvate, NADH, NGF (7S), alphidicolin, TPA were from Sigma Aldrich. SB203580 was from Calbiochem. The Dual luciferase assay kit and β-galactosidase assay kit were from Promega. Acrylamide/Bis and non-fat milk were from Bio-Rad. DEVD-afc and z-DEVD-fmk were obtained from BACHEM. Other chemicals or reagents are individually mentioned in the following sections where are relevant.

All antibodies used in the research are listed in table 2.1.

Table 2.1 Antibodies used in the research

Name	Host/Clone	Source
c-Jun	Rabbit/polyclonal	Cell Signaling
Phospho c-Jun (Ser 63)	Rabbit/polyclonal	Cell Signaling
Phospho c-Jun (Ser 73)	Rabbit/polyclonal	Cell Signaling
JNK	Rabbit/polyclonal	Cell Signaling
Phospho JNK	Rabbit/polyclonal	Cell Signaling
actin	Goat/polyclonal	Santa Cruz
SgII	Goat/polyclonal	Santa Cruz
Myc	Mouse/monoclonal	Invitrogen
Bcl-2	Mouse/monoclonal	Transduction Laborotories
Caspase 3	Rabbit/polyclonal	Pharmingen
PARP	Mouse/monoclonal	Pharmingen

NCAM	Mouse/monoclonal	Chemicon

2.2 Cell culture

SH-Sy5y and SHEP cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 4.5 g/L glucose, 10% (v/v) fetal bovine serum (FBS, Hyclone), 100 units/ml penicillin and 100 ng/ml streptomycin (Sigma) in a humidified incubator containing 5% CO_2. Stable cells were selected and maintained in hygromycin (BD Bioscience), G418 (Calbiochem) or zeocin (Invitrogen) accordingly. (See Table 2.2)

2.3 Transfection of mammalian cells

2.3.1 Transcient transfection using LIPOFECTIN

Approximately 1-2 × 10^5 cells were seeded onto 6-well or 35-mm tissue culture plates 16 h before transfection. 6 µl of LIPOFECTIN (Invitrogen) was diluted into 100 µl of opti-MEM medium (Invitrogen) and incubated at room temperature for 30-35 min. 1-2 µg of plasmid DNA was diluted into 100 µl of opti-MEM medium and combined together with the LIPOFECTIN-opti-MEM mixture. The DNA-LIPOFECTIN mixture was incubated at room temperature for 15 min and topped up with opti-MEM to 1 ml. Cells were rinsed with opti-MEM medium followed by incubation with the transfection mixture for 8-10 h at 37ºC. Subsequently, the transfection mixture was removed and the cells were incubated for another 40 h in complete growth medium before treatment.

2.3.2 Stable transfection using LIPOFECTIN

Approximately 6×10^5 cells were seeded onto 50mm tissue culture plates 16 h before transfection. 12 µl of LIPOFECTIN was diluted into 100 µl of opti-MEM medium and incubated at room temperature for 30-35 min. 5 µg of plasmid DNA was diluted into 100 µl of opti-MEM medium and combined together with the LIPOFECTIN-opti-MEM mixture. The DNA-LIPOFECTIN mixture was incubated at room temperature for 15 min and top-up with opti-MEM to 2 ml. Cells were rinsed with opti-MEM medium followed by incubation with the transfection mixture for 8-10 h at 37ºC. Subsequently, the transfection mixture was removed and the cells were incubated for 24 h in complete growth medium. After that, the medium was refreshed with selection medium. Selection medium was replaced every 2 or 3 days. About 3 weeks later, resistant clones were picked up and cultured for further identification.

Table 2.2 Stable cell lines used in the current study

Cell lines	**Medium**	**Description**	**Source**
TAM67	DMEM/H	SH-Sy5y cells stably transfected with TAM67 plasmids	Dr. Zhiwei Feng
JunAA /S63A/S73A	DMEM/H	SH-Sy5y cells stably transfected with JunAA/S63A/S73A plasmids	Own construction
DN-*jnk1*/*jnk2*	DMEM/G	SH-Sy5y cells stably transfected with DN-*jnk1*/*jnk2* plasmids	Own construction
Si-*sgII*	DMEM/H	SH-Sy5y cells stably transfected with *sgII*- siRNA plasmids	Own construction
T-S	DMEM/H,Z	TAM67 cells stably transfected	Own construction

		with *sgII* expression plasmids	
T-7B2	DMEM/H,Z	TAM67 cells stably transfected with 7B2 expression plasmids	Own construction

G: G418 at 100 μg/ml; H: Hygromycin at 50 μg/ml (TAM67) or 100 μg/ml (the others), Z: Zeocin at 100 μg/ml. DMEM: Dulbecco's Modified Eagle Medium.

2.4 Molecular cloning

2.4.1 Construction of expression plasmids

The plasmid encoding dominant-negative c-Jun (denoted JunAA) was obtained from Dan Mercola (Sidney Kimmel Cancer Center, San Diego, CA, USA). The plasmids bearing the dominant-negative S63A or S73A mutations in c-Jun were constructed by PCR-based mutagenesis based on the plasmid JunAA. The primers used were: S63A (F) 5'-GCT CAA GCT GGC GTC TCC CGA GCT GG-3'; S63A (R) 5'-CCA GCT CGG GAG ACG CCA GCT TGA GC-3'; S73A (F) 5'-CCT CCT CAC CTC TCC CGA CG-3'; S73A (R) 5'-CGT CGG GAG AGG TGA GGA GG-3', respectively. The Gal4-c-Jun transactivator and Gal4-luciferase reporter plasmids were purchased from Stratagene. The modified Gal4-c-Jun plasmids (S63A, S73A or JunAA) were constructed by cloning the transactivation domain of c-Jun (amino acids 1-221) bearing the S63A, S73A or JunAA mutations into the pFA-CMV vector from Stratagene. The pgl3-TRE and pgl3-CRE reporter plasmids and RPL-TK plasmid were kindly provided by S. Dhakshinamoorthy (Institute of Molecular and Cell Biology, Singapore). The plasmid encoding dominant-negative (DN)-JNK1 was provided by Roger J. Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA), and the plasmid encoding DN-JNK2 was provided

by Dr. Shengcai Lin (Institute of Molecular and Cell Biology, Singapore). The plasmids encoding dominant-negative c-Jun (denoted TAM-67) was provided by R. Jaggi (University of Bern, Bern, Switzerland). The primers used for amplification and cloning of the *sgII* promoter were: (F) 5'- CCG GTA CCG TAC GAA GCT TCC TTT CGA TTG CAA ATG AAT TTC-3'; (R) 5'- CCG CTC GAG GCT CCA CAG CAT ATT CCT CCC GTT CTC CGG G-3'. 200 ng genomic DNA from SH-Sy5y cells was used for amplification of the *sgII* promoter with expand Hifi DNA polymerase (Roche). The PCR product was cloned into pgl3-basic and pgl3-enhancer vectors (Promega) at *Kpn*I and *Xho*I sites. The primers used for mutagenesis of CRE motif in *sgII* promoter were: (F) 5'-GCT GAA CCC GGA GTG GTC AGT GTG GC-3'; (R) 5'-GCC ACA CTG ACC ACT CCG GGT TCA GC-3'. The mutant CRE motif is underlined.

2.4.2 Preparation of *Escherichia coli* competent cells

The DH5α, XL-blue *E. coli* strain was streaked onto an LB (pH 7.5) agar plate (containing 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, and 1.5% agar) without antibiotics and incubated at 37°C overnight. A single colony was inoculated into 30 ml of LB and grown at 37°C with shaking. The overnight culture was added to OD_{600} 0.1 in 400 ml of LB and grown for approximately 3 h at 37°C to reach OD_{600} 0.3-0.6. The bacterial culture was harvested by centrifugation at 4000 rpm for 10 min at 4°C. The pellet was washed on ice in 100 ml of 100 mM $CaCl_2$. Subsequently, the bacteria were centrifuged again at 4000 rpm for 10 min at 4°C. The pellet was resuspended in 8 ml of 100 mM $CaCl_2$ with 10% glycerol. The competent cells were aliquoted and stored at –80°C.

2.4.3 DNA transformation

The ligated DNA (5 μl, about 100 ng) or pure plasmid DNA (about 10 ng) was mixed with 100 μl of competent cells, which were pre-thawed on ice. After the mixture was incubated on ice for 30 min, the cells were heat-shocked for 90 seconds at 42°C followed by placing on ice for 10 min. For ampicillin-resistant plasmids, the cells were directly spread onto the LB agar plates containing 50 μg/ml ampicillin. For plasmids with kanamycin resistance genes, 1 ml of LB was added to the heat-shocked cells followed by shaking at 37°C for 1 h. Before the cells were spread onto LB plates containing the appropriate antibiotic, a brief spin of the cells in a micro-centrifuge was performed to reduce the volume.

2.4.4 DNA preparation

Small-scale plasmid DNA isolation from *E. coli* cultures was carried out using the Rapid Plasmid Miniprep System (Marligen Bioscience, MD, USA) according to the manufacturer's instruction. The overnight bacterial culture was harvested by centrifugation at 14,000 rpm for 5 min, and the supernatant was discarded. The cell pellet was completely resuspended in Cell Resuspension Solution [50 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 100 μg/ml RNase A], followed by the addition of Cell Lysis Solution (0.2 M NaOH and 1% SDS), Alkaline Protease Solution and Neutralization Solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate and 2.12 M glacial acetic acid, pH 4.2). The bacterial lysate was centrifuged at 14,000 rpm for 10 min at room temperature, and the cleared lysate was transferred to a Spin Column. After centrifugation for 1 min, the Wash Solution, diluted with 95% ethanol, was added to the Spin Column (162.8 mM potassium acetate and 27.1 mM Tris-HCl,

pH 7.5), followed by another centrifugation. The wash procedure was repeated again before the plasmid DNA was eluted in an appropriate amount of TE or nuclease-free water.

Large-scale plasmid DNA isolation was carried out by using the QIAGEN Plasmid Maxi Kit (QIAGEN) according to the manufacturer's instructions. Similar to the miniprep process, large volumes of bacterial culture were centrifuged into pellets and subsequently incubated with the resuspension, lysis and neutralization buffer sequentially. After clearing of the bacterial lysate by centrifugation, the supernatant was applied to a the pre-equilibrated QIAGEN-tip 500 column, followed by washing with wash buffer (1 M NaCl, 50 mM MOPS pH 7.0 and 15% isopropanol). The plasmid DNA was eluted by elution buffer (1.25 M NaCl, 50 mM TrisCl pH 7.0 and 15% isopropanol) and precipitated by 0.7 volumes of isopropanol. The DNA pellet was washed with 70% ethanol, air-dried, and redissolved in a suitable volume of TE buffer or nuclease-free water.

Genomic DNA isolation was carried out using the QIAGEN DNA mini kit. Cells from one 80 cm^2 flask (NUNC, Denmark) were harvested, washed once with ice-cold PBS and spun down at 2000 rpm for 5 min at 4ºC. The cells were resuspended in 1 ml of PBS. 1 ml of ice-cold lysis buffer (1.28 M sucrose; 40 mM Tris·Cl, pH 7.5; 20 mM $MgCl_2$; 4% Triton X-100) and 3 ml of H_2O were added into the cell suspensions. The mixture was incubated on ice for 10 min and subjected to centrifugation at 2000 rpm for 15 min at 4ºC. The supernatant was discarded and the pellets were resuspended in 0.5 ml of lysis buffer and 1.5 ml of H_2O. The mixture was vortexed and centrifuged at 2000 rpm for 15 min at 4ºC. The supernatant was discarded and 1 ml of digestion buffer (800 mM guanidine HCl; 30 mM Tris-Cl, pH 8.0; 30 mM EDTA, pH 8.0; 5% Tween-20; 0.5% Tritin X-100) was added to resuspend the pelleted nuclei.

The nuclei were vortexed at maximum speed for 30 sec. 50 µl of protease was added into the nuclei followed by the incubation at 50ºC for 30 min. The sample was vortexed after lysis for 10 sec at maximum speed and applied to the pre-equilibrated QIAGEN genomic tip. The tip was washed with 3 × 1 ml of wash buffer (1.0 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol) and the genomic DNA eluted with prewarmed elution buffer (1.25 mM NaCl; 50 mM Tris.Cl, pH 8.5; 15% isopropanol). The DNA was precipitated by adding 0.7 volume of isopropanol to the eluted DNA and spun down. The DNA was air-dried and dissolved in TE or nuclease free H_2O.

2.5 Polymerase chain reaction (PCR)

PCR for cloning purposes was performed by using the expand Hifi DNA polymerase (Roche) and the primers of the required sequences were synthesized by Genset Inc., Singapore. Typically, to amplify DNA fragments, 20-50 ng of cDNA template in the PCR buffer was supplemented with 1.75 mM $MgCl_2$, and mixed with 250 µM of the deoxy-adenine, deoxy-cytosine, deoxy-guanine and deoxy-thymidine nucleotides (QIAGEN), 50 pM of the sense and anti-sense primers, and 2.5 Units of the Hifi DNA polymerase (Roche). The reaction was carried out by 1 cycle of denaturation at 94°C for 2 min and primer annealing at 50-60°C for 30 sec (dependent on the primers used), followed by 30 cycles of DNA extension at 68°C for up to 2 min (dependent on the length of the PCR product), denaturation at 93°C for 15 sec, and primer annealing at 50-60°C for 30 sec. A final cycle of DNA extension was carried out at 68°C for 7 min. For the PCR reaction for non-cloning purpose, Taq DNA polymerase (QIAGEN) was used.

2.6 Site-directed mutagenesis

Point mutations were introduced into the plasmid cDNAs using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Complementary primers with point mutations were designed and used for extension in temperature cycling to generate mutant plasmids from the wild-type plasmid template. The temperature-cycling conditions involved 16 cycles of denaturation at 95°C for 30 sec, primer annealing at 50°C for 1 min, and extension at 68°C for 14 min (based on 2 min/kb of plasmid length). The methylated wild-type template was removed from the newly synthesised, non-methylated plasmids by *Dpn*I (New England Biolabs) restriction digest at 37°C for 1h. The mutant plasmids were transformed in *E. coli* for clonal isolation and the mutations were confirmed by standard dideoxy termination sequencing.

2.7 Sytox/Hoechst DNA staining

S/H staining is an easy and quick way to distinguish between necrosis (passive cell death) and apoptotis (active, programmed cell death). Hoechst-33342 (Molecular Probes) is a blue membrane permeable DNA dye and sytox (Molecular Probes) is a green membrane impermeable DNA dye. When applied to cells: normal, healthy control cells with intact cell membranes display round and blue nuclei, whereas apoptotic (Sytox-impermeable membrane, only H-33342-stained) show blue strongly condensed/fragmented nuclei. In contrast, necrotic cells (damaged and therefore Sytox-permeable membrane) appear swollen-greenish.

After treatment, the Sytox/ Hoechst stain was added into the cell culture medium at final concentration of 500 nM and 500 ng/ml respectively and the cells were incubated at 37˚C for up to 5 min. Cells were then visualized under the Zeiss

epifluorescence microscope, and photos were taken with an attached Nikon Coolpix digital camera.

2.8 Cell death assay

Three different methods assaying cell death based on different principles were used in the research. Although the absolute values might be different by using these different methods, the pattern of death was similar. LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into cell culture medium when the plasma membrane is damaged. So the percentage of LDH release is proportional to percentage of damaged cells. WST is a tetrazolium salt which can be cleaved by mitochondria enzymes in metabolically active cells to form water-insoluble formazen which can be directly measured in an ELISA plate reader. So the healthier the cells are, the higher WST reading will be. Crystal violet staining is an assay for cell attachment. For adherent cells, when cell death occurs, the cells will become detached thus cannot be stained due to the extensive wash step.

LDH (lactate dehydrogenase) release assay. Cells (1-2 $\times$ 10^4 /well) were plated in 96-well plates and subjected to the desired treatments. After the treatment, the supernatants of the cells were collected, and the cell layer was lysed with an equal volume of lysis buffer (DME plus 0.1% Triton X-100). LDH activity in the supernatant and the lysate was quantitated. The cytotoxicity was calculated as percentage of LDH release by the ratio of supernatant/ (lysate + supernatant).

WST assay. Cells (1-2 $\times$ 10^4 /well) were plated in 96-well plates and subjected to the desired treatments. After treatment, WST was added into each well at 1:10 dilution.

The cells were further incubated in a 37ºC incubator until the color changed and subjected to measurement at 420-480 nm.

Crystal violet staining. Cells (1-2 × 10^4 /well) were plated in 96-well plates and subjected to the desired treatments. After the treatment, the cell culture medium was aspirated and cells were stained with a solution (20% methanol, 0.5% crystal violet) for up to 15 min at room temperature. The excess stain was removed completely by washing with distilled water many times. The cells were left to air-dry, and the stain was solubilized in 33% acetic acid solution. The absorbance was measured at 590 nm.

2.9 Reporter assay

For the Gal4-c-Jun reporter assay, 50 ng of Gal4-c-Jun activator plasmids (wild type, S63A, S73A or JunAA), 1 μg of Gal4-luciferase reporter plasmid and 10 ng of β-galactosidase plasmid were co-transfected into SH-Sy5y cells in 6-well plates (1-2 × 10^5 cells/plate). 40 h later, cells were treated with 2 mM SNP for the indicated times. Medium was removed and cells were washed three times with ice-cold PBS. Cells were harvested with 400 μl reporter lysis buffer (provided in the β-gal assay kit) and subjected to a short spin. 20 μl of supernatant was added to 100 μl luciferase substrate and the luciferase activity was immediately measured in a TD-20e luminometer. An aliquot of the same sample was used to determine β-galactosidase activity for normalizing luciferase activity obtained above.

For the AP-1 reporter assay, 1 μg of pGL3-TRE or pGL3-CRE reporter plasmid and 10 ng of RPL-TK plasmid were cotransfected in cells seeded on 6-well plates. 40 h later, cells were either harvested directly or treated with 2 mM SNP for the indicated times and harvested as described above. Lysates were diluted ten times, and firefly luciferase activity was measured by adding 10 μl of diluted lysate and 100 μl of

firefly luciferase substrate. The *Renilla* luciferase activity, as internal control, was measured by adding Stop&Glo solution in the same tube.

For the *sgII* promoter analysis, 1 μg of pGL3-*sgII promoter* reporter plasmids and 10 ng of RPL-TK plasmid were co-transfected cells seeded on 6-well plates. 40 h later, cells were either harvested directly or treated with 2 mM SNP for various times and harvested. Luciferase activity was measured as described above.

2.10 Caspase-3 activity assay

The activity of caspase-3-like proteases was measured using microtiter plates as described with modifications (Hentze *et al*., 2002). After SNP treatment, the cells were lysed in lysis buffer (20 mM Hepes, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1μg/ml each pepstatin, leupeptin and aprotinin), and the lysate was stored at –80°C if not for immediate use. The samples were diluted 1:10 with reaction buffer (60 μM fluorogenic substrate DEVD-afc in 50 mM HEPES, pH 7.4, 1% sucrose, 0.1% CHAPS, 10 mM DTT) in a final volume of 100 μl and incubated at 37°C for 30 min. Released afc was kinetically measured with a fluorescent spectrophotometer set at excitation wavelength of 400 nm and emission wavelength of 505 nm. For normalization, protein concentrations of the corresponding samples were estimated simultaneously by using the BCA reagents from Pierce Chemical Company. 1 μU/mg corresponds to 1 (μmol/mg) × min.

2.11 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The Bio-Rad Mini-PROTEAN II system was used for routine protein gel electrophoresis. The resovlving gels included 6-15% acrylamide (29:1) (Bio-Rad), 0.375 M Tris-HCl pH 8.8 and 0.1% (w/v) sodium dodecyl sulfate (SDS), and were

polymerized by the addition of freshly prepared 0.1% (w/v) ammonium persulfate and 0.01% (v/v) TEMED. The stacking gels included 4% acrylamide, 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS, and were polymerized as described for the separating gels. Protein samples were mixed with an equal volume of 2X protein sample buffer [2X: 125 mM Tris-HCl (pH 6.8), 4% SDS (v/v), 10% β-mercaptoethanol (v/v), 20% glycerol (v/v) and 0.4% bromophenol blue (w/v)], and heated at 95°C for 5 min before loading into the wells. Electrophoresis buffer consisted of 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 0.1% SDS. The gels were subjected to electroblotting onto polyvinylidene difluoride (PVDF) membrane (Millipore) for Western blot analysis.

2.12 Western blot analysis

After SDS-PAGE, the resolved proteins were electroblotted onto a PVDF membrane (pre-activated with methanol) using the Bio-Rad Trans-Blot^{TM} system. Electroblotting was carried out in the transfer buffer composed of 25 mM Tris-base, 192 mM glycine, and 20% methanol for 1 h at 4°C. The membrane was then incubated in blocking buffer [PBS containing 5% (w/v) non-fat milk (Bio-Rad) or BSA and 0.1% (v/v) polyoxyethylene-sorbitan monolaurate (Tween-20, Sigma)] for at least 1 h. The membrane was then incubated with the relevant antibodies diluted in blocking buffer to the optimized working concentrations for overnight incubation at 4ºC. Unbound antibodies were removed by extensive washing in PBS containing 0.1% Tween-20. After incubation with the secondary antibody for about 2 h at room temperature and washing with PBS containing 0.1% Tween 20, the membrane was then developed using an enhanced chemiluminescence (ECL) kit.

For reprobing of the same membrane with a different antibody, the immunoblot was incubated in stripping buffer [62.5 mM Tris (pH 6.8), 100 mM β-mercaptoethanol,

and 2% SDS] for 30 min at 60°C to remove the previous antibody. The membrane was then re-blocked with blocking buffer and incubated with another antibody.

2.13 Phospho-Jun and -JNK assay

Cells were harvested after different times of 2 mM SNP treatment and lysed in 200 µl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromphenol blue and 50 mM freshly added DTT). Sonication was needed to shear the genomic DNA and reduce the viscosity of the lysates. The sonicated lysate was then heated at 99°C for 5 min and subjected to centrifugation at 14,000 rpm for 5 min at 4°C. 50 µl of the supernatants was separated on 12% of polyacrylamide gels and transferred onto PVDF membranes (Millipore). Detection of bands was performed using the Phototope®-HRP Western Blot Detection System (Cell Signaling).

2.14 Peptide inhibition assay

The inhibition assay was carried out in SHEP neuroblastoma cells. D-TAT and D-JNKI1 peptides (Alexis Biochemicals, Switzerland) were added at final concentrations of 20, 50 and 100 uM into the medium. After 24 h, the medium was refreshed with peptides, and SNP was added at final concentration of 2 mM. At various times thereafter, cell death and c-Jun phosphorylation were measured as described above.

2.15 RNA preparation

Cells grown in monolayer were lysed directly in a culture dish. The media was decanted, TRI REAGENT was added, and the cell lysate was passed several times through a pipette. 1 ml of TRI REAGENT (Invitrogen) was used per 10 cm^2 of culture

dish area. The homogenate was stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2ml chloroform was added into the 1 ml of homogenate and the mixture was vortexed for 15 sec. The mixture was stored in room temperature for 15 min and subjected to centrifugation at 12,000 g for 15 min at 4ºC. After centrifugation, the supernatant was carefully transferred into a fresh tube. RNA from the supernatant was precipitated by mixing with isopropanol (Use 0.5 ml of isopropanol per 1 ml of TRI REAGENT used for the initial homogenization.). The samples were stored at room temperature for 10 min and centrifuged at 12,000 g for 8 min at 4ºC. The supernatant was removed, and the RNA pellet was washed with 75% ethanol and centrifuged at 7,500 g for 5 min at 4ºC. The ethanol wash was removed and the RNA pellet was air-dried for 3-5 min at room temperature. RNA was finally dissolved in RNase-free H_2O and frozen for further use.

Total RNA (less than 250 µg) was diluted with RNase-free water to 250 µl, incubated with 15 µl of Oligotex resin (QIAGEN) and 250 µl of OBB. The mixture was thoroughly mixed by pipetting and incubated at 70ºC for 3 min followed by an incubation at room temperature for 10 min, allowing the hybridization between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA. Oligotex:mRNA complexes were collected by centrifugation for 2 min at maximum 14,000 g. The supernatant was carefully removed by pipetting and the pellet was resuspended in 400 µl of buffer OW2 by votexing, added onto a small spin column (supplied in the kit) which was placed in a 1.5 ml microcentrifuge tube and centrifuged for 1 min at maximum speed (14,000 rpm). The spin column was transferred to a new RNase-free 1.5 ml microcentrifuge tube, and 400 µl of buffer OW2 was applied to the column. A short centrifugation was performed for 1 min at maximum speed, and the flow-through was discarded. The spin column was transferred to a new RNase-free 1.5 ml

microcentrifuge tube and 50 µl hot (70ºC) buffer OEB was pipetted onto the column, and 3-4 up and down pipetting steps were applied to resuspend the resin. A final centrifugation was carried out for 1 min at maximum speed to elute the mRNA. To concentrate the mRNA obtained, a precipitation step may be required. mRNA (at the starting volume of 50 µl) was mixed with 40 µl of 1×TE, 1 µl of glycogen (5 mg/ml), 10 µl of 3M NaAc (pH 5.2) and 250 µl of 100% ethanol. The mixture was frozen at -80ºC for 20 min and subject to centrifugation for 20 min at 12,000 g at 4ºC; the supernatant was removed and 250 µl of 75% ethanol was applied to wash the pellet. Centrifugation at 12,000 g for 5 min was performed, the supernatant was removed and the pellet was air-dried. The pellet was dissolved in an appropriate volume of RNase-free water and frozen for further use.

2.16 Microarray analysis

SH-Sy5y cells and TAM67 stable cells were treated with 2 mM SNP for 6h or 10h, total RNA was extracted using Trizol reagent (Invitrogen) and mRNA was purified using the Oligotex^{TM} mRNA kit (QIAGEN). Details are described in 2.16. The mRNA concentration was measured and its integrity was confirmed by PCR amplification of randomly selected genes. Microarray analysis was carried out by Incyte Genomics (St. Louis, MO, USA) under contract.

2.17 Induction of neuronal differentiation

Differentiation was induced as described with modifications (Jensen *et al*., 1992). Cells were plated on 60 mm tissue culture dishes at a density of 5-6×10^5 cells/dish. After overnight culturing, nerve growth factor (NGF-7S, Sigma) was added to the medium at a final concentration of 1µg/ml. On the second day, the medium was

refreshed with 5 μg/ml aphidicolin (Sigma) in the same medium. On the third day, the medium was refreshed with 1μg/ml NGF and 5 μg/ml alphidicolin. The medium was refreshed every two days with NGF and alphidicolin for 8-10 days.

2.18 Preparation of whole cell lysates

Cells were washed once with ice-cold PBS and lysed in lysis buffer [50 mM Tris·Cl, pH 8.0; 150 mM NaCl; 0.02% sodium azide; 1% Triton X-100 and freshly added protease inhibitor (Roche Applied Science)] on ice for 30 min and subjected to centrifugation at 14,000 rpm for 30min at 4°C. The supernatant was collected and the protein concentration was measured.

2.19 Preparation of nuclear extracts

Cells were harvested and washed twice with ice-cold PBS. Cell pellets were resuspended in buffer A (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and complete protease inhibitors) and homogenized in a Dounce homogenizer. After centrifugation at 600 g for 20 min, the pellets containing nuclei (supernatants, the cytoplasmic proteins, may be kept for further use) were resuspended in buffer C (20 mM HEPES [pH 7.9, 1.5 mM MgCl2, 420 mM KCl, 1 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, and complete protease inhibitors) and incubated on ice for 20 min. The samples were centrifuged at 100,000 g for 20 min at 4ºC, the supernatants were collected as nuclear proteins.

2.20 Detection of proteins released into the cell culture medium

To detect protein release in the cell culture medium, serum-free culture medium was used to reduce background proteins. After treatment, the cell culture medium was

carefully collected and 50 µl of StrataClean™ resin was added into the medium followed by vortexing for 15 sec. The mixture was centrifuged for 2 min at maximum speed at room temperature and supernatants were removed by pipetting. Pellets were resuspended in 50 µl of SDS sample buffer and equal volumes of samples were loaded on SDS-PAGE gels followed by western blot analysis.

2.21 Eletrophoretic Mobility Shift Assay (EMSA)

Reactions were conducted in a total volume of 20 µl. Typically, 5 µl of nuclear extracts (about 5 µg of protein) was added to the reaction buffer containing 100 mM KCl, 10% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg of leupeptin/ml, 20 mM HEPES (pH 7.9), and 2 µg of poly (dI-dC). The mixture was incubated on ice for 20 min, and 10^5 cpm (3,000 Ci/mmol) of [γ-^{32}P] ATP-labeled oligonucleotide probe with an AP-1 binding site (5'-CGC TTG ATG ACT CAG CCG GAA-3') was added. The oligonucleotide-protein mixture was incubated on ice for another 20 min and loaded on the 5% native polyacrylamide gels in Tris-glycine buffer (pH 8.5) for 3 h at 4ºC. The gels were dried and autoradiographed with intensifying screen at -80ºC.

2.22 Annexin V staining

Cells were treated with SNP for 16 h, harvested and washed twice with ice-cold PBS; and then were resuspended in 1X Binding Buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$) at a concentration of ~1 x 10^6 cells/ml. Transfer 100 µl of the solution (~1 x 10^5 cells) to a 5 ml culture tube. 5 ul of Annexin V-FITC (Pharmingen) was added into the tube followed by gentle mixing and incubation for 15

min at room temperature in the dark. 400 µl of 1X Binding Buffer was added to each tube and sample was analyzed by flow cytometry without delay (within 1 hr).

2.23 Semi-quantitative RT-PCR analysis

SH-Sy5y and TAM67 stable cells were treated with 2 mM SNP for different times, total RNA was prepared (described in 2.16), followed by reverse transcription with Superscript II polymerase (Invitrogen Life Technologies) and PCR amplification with Tag DNA polymerase (QIAGEN). The PCR temperatures consisted of a 5 min denaturation step at 95 °C, followed by the indicated numbers of cycles at 95 °C for 30 s, annealing for 30 s, and 72 °C for 45 s or 1.5 min according to the size of the amplified fragments. The numbers of PCR cycles, annealing temperatures and primers used were as follows.

SgII, 25 cycles, annealing temperature 55 ˚C, forward: 5'-CGA CGG GAT CCA CCA TGG CTG AAG CAA AGA CCC ACT GGC TTG GAG-3'; reverse: 5'-GCA GCA CTC GAG CAT ATT TTC CAT TGC TCT CTT AGC AAT ATG C-3'. The primers were also used for cloning of SgII into pcDNA4/*myc*-His mammalian expression vector (Invitrogen Life Technologies).

CGA, 25 cycles, annealing temperature 60 ˚C, forward: 5'-GCG CAA GCT TGC CAC CAT GCG CTC CGC CGC TGT CC-3'; reverse: 5'-GCG CGA ATT CGC CCC GCC GTA GTG CCT GC-3'.

CGB (SgI), 35 cycles, annealing temperature 55 ˚C, forward: 5'-CGA CGA AGC TTG CCA CCA TGC AGC CAA CGC TGC TTC TCA GCC-3'; reverse: 5'-GCA GGG ATC CGC CCC TTT GGC TGA ATT TCT CAG C-3'.

7B2, 25 cycles, annealing temperature 55 ˚C, forward: 5'-CGA CGA AGC TTG CCA CCA TGG TCT CCA GGA TGG TCT CTA CC-3'; reverse: 5'-GCA GGA ATT

CCT CTG GAT CCT TAT CCT CAT C-3'. The primers were also used for cloning of SgII into pcDNA4/*myc*-His mammalian expression vector.

Actin, 20 cycles, annealing temperature 55 ˚C, forward: 5'-GAT GCA TTG TTA CAG GAA GT-3'; reverse:5'- TCA TAC ATC TCA AGT TGG GGG-3'.

2.24 RNA interference

Six different oligonucleotide sequences were chosen for the siRNA-mediated knock-down of *SgII* using the siRNA target finder software at www.ambion.com. The hairpin siRNA-encoding oligonucleotides were synthesized along with the loop sequence TTCAAGAGA and the *Bam*H I and *Hind* III restriction sites (added to the 5' and 3' end of the DNA oligonucleotides respectively) and cloned into the siRNA vector p*Sillencer*^{TM} 2.1-U6 (Ambion, Austin TX) followed by sequencing.

The six oligonucleotides are listed in table 2.3.

Table 2.3 Lists of oligonucleotides used for *sgII* knock-down

	Starting Position in *SgII* mRNA	**Sequence (5' to 3')**
1	551	(AA)ATAGTGGAGGAACAATATA
2	619	(AA)ACTGACAGGACCAAACAAC
3	823	(AA)TGAACAAATCAACGATGAG
4	1492	(AA)CGACAAGGATCAAGAATTA
5	1622	(AA)CAAATTGAGCAGGCCATCA
6	1726	(AA)TGATGATACCCCAAATAGG

CHAPTER 3 JNK-dependent phosphorylation of c-Jun on Ser-63 mediates NO-inducible apoptosis in human SH-Sy5y neuroblastoma cells

NO is a small, diffusible molecule with important physiological relevance such as regulation of neurotransmission and involvement in non-specific host defense against pathogens. There is accumulating evidence that excessive NO generation during strokes, ischemia or neurodegenerative diseases also contributes to neuronal cell death (Coyle and Puttfarcken, 1993; Torreilles *et al*., 1999). NO can exert its cytotoxic effects in diverse cell types *via* generation of highly reactive free radicals like peroxynitrite, which triggers downstream signal transduction pathways and lead to apoptosis or necrosis. However, the death pathways that are activated in neurons in response to massive NO production are not well understood.

3.1 NO induces concentration-dependent apoptosis in SH-Sy5y cells

Various NO donors have been widely used to study oxidative stress and cellular responses by mimicking endogenous NO generation (Brune *et al*., 1999). Many neuronal cell lines, including SH-Sy5y neuroblastoma cells, are highly sensitive to cell death induced by different NO donors (Feng *et al*., 2002n; Ghatan *et al*., 2000d; Oh-Hashi *et al*., 1999). The mode of cell death under those circumstances can be either apoptosis or necrosis depending on the concentrations of the NO donors used. In the current study, SH-Sy5y cells were treated with different concentrations of SNP (a NO donor) for 15 hr and stained with Hoechst 33342 (a cell permeable dye for nucleic acid staining) plus Sytox (a non-permeable dye for nucleic acid staining). An obvious

nuclear condensation or chromatin fragmentations was observed at SNP concentrations higher than 1.5 mM (Fig 3.1A). Consistent with the above results, caspase-3 cleavage and activation, and PARP cleavage were also detected after a few hours treatment with 2 mM SNP (Fig 3.1B). As DNA fragmentation, caspase-3 activation and PARP cleavage are the hallmarks of apoptosis, it can be concluded that under these experimental conditions, SNP-treated SH-Sy5y cells died by apoptosis.

3.2 JNK activation correlates with c-Jun phosphorylation of Ser-63 in response to NO during apoptosis in SH-Sy5y cells

A time-dependent increase in cell death was observed beginning around 8 h after addition of SNP to the SH-Sy5y cells (Fig 3.2A), and this increase correlated with the appearance of significant JNK kinase activity at 6 to 7.5 h after addition of SNP (Fig 3.2B, *upper panel*). JNK phosphorylation seemed to occur majorly at 46 KDa isoform instead of 55 KDa isoforms, the significance of which was not clear. Since JNKs are the main upstream kinases for c-Jun NH_2-terminal phosphorylation (Karin, 1995), I next tested whether the Ser-63 or Ser-73 residues of c-Jun were phosphorylated after SNP treatment by using specific antibodies detecting phosphoserine-63 or phosphoserine-73. A strong and sustained c-Jun phosphorylation on Ser-63 was observed at 6-8 h after the addition of SNP, whereas phosphorylation on Ser-73 was virtually undetectable (Fig 3.2C, *upper and middle panel*). In contrast, UV irradiation of SH-Sy5y cells resulted in similar levels of Ser-63 and Ser-73 phosphorylation (Fig 3.2C). Thus, JNK activation and selective phosphorylation of c-Jun on Ser-63 both occurred around the onset of NO donor-induced apoptosis. Ser-63 phosphorylation and the death of SH-Sy5y cells both occurred at 1.5 mM SNP or higher. Concentrations of SNP lower than 1.5 mM induced neither cell death (Fig 3.1A and Fig 3.2E) nor Ser-63

phosphorylation of c-Jun (Fig 3.2D), indicating that c-Jun is phosphorylated only at the toxic concentrations of SNP. Excessive concentrations of SNP higher than 2.5 mM resulted in detectable Ser-73 phosphorylation that closely correlated with the onset of appreciable necrosis, indicating that predominant Ser-63 phosphorylation is an apoptosis-related phenomenon.

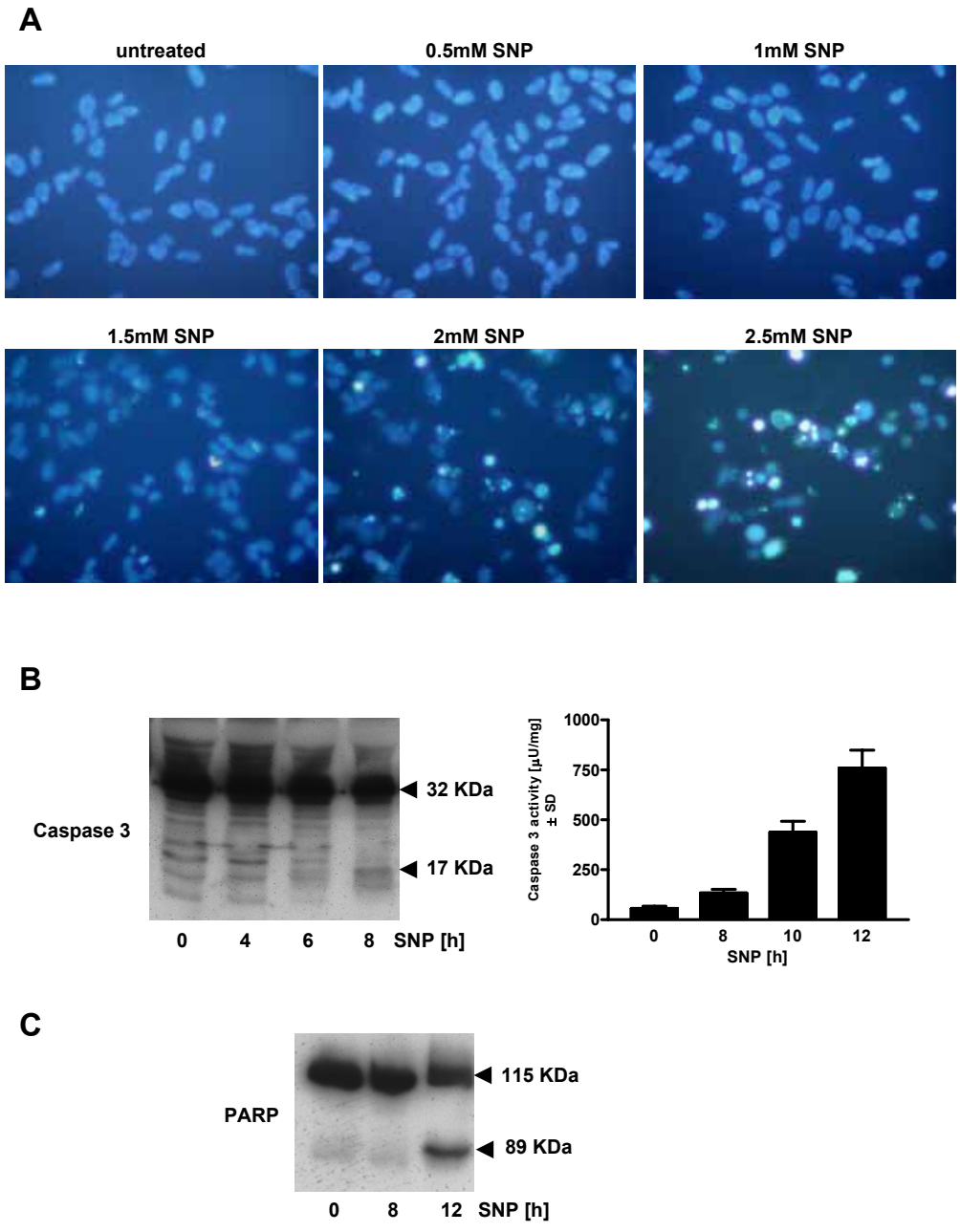

Figure 3.1. NO induces concentration-dependent apoptosis in SH-Sy5y cells. (A) Nuclear staining: SH-Sy5y cells were treated with different concentrations of SNP for 15 h, Sytox/Hoechst 33342 was added and cells were visualized as described in the "materials and methods". SH-Sy5y cells were treated with 2 mM SNP for different times, cells were harvested and whole cell lysates were prepared. Caspase-3 cleavage (Fig B, *left panel*) and PARP cleavage (Fig C) were detected. Caspase-3 activation was also detected as described in the "materials and methods" (Fig B, *right panel*).

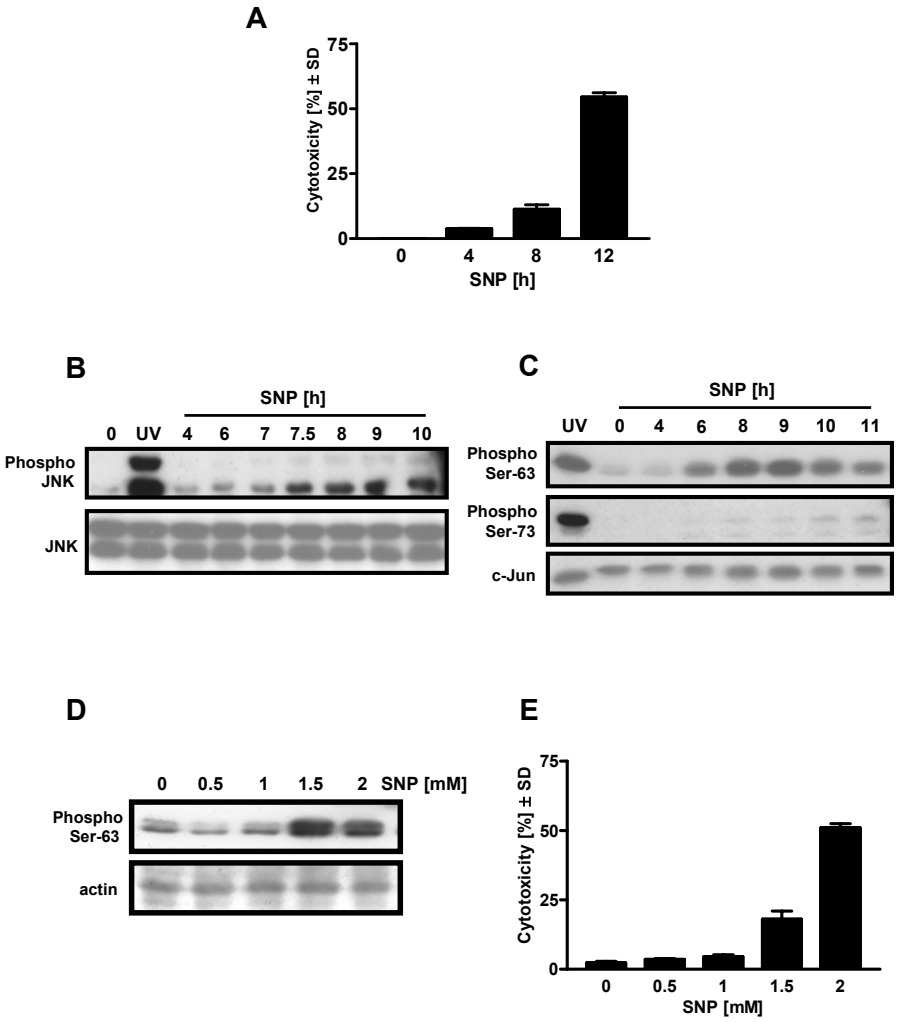

Fig 3.2 JNK activation correlates with c-Jun phosphorylation of Ser-63 in response to NO during apoptosis in SH-Sy5y cells. (A) SH-Sy5y cells were treated with 2 mM SNP for different times and

harvested, the percentage of dead cells was measured by LDH release assay as described in the "materials and methods". Cells were treated with UV at 100 J/m^2 following by 1 h incubation at 37ºC or 2 mM SNP for different times, whole cell lysates were prepared and subjected to 12% SDS-PAGE gel and Western Blot analysis, detecting the phosphorylation of JNK (B) and c-Jun (C). JNK and c-Jun protein were revealed as a loading control. (D) Cells were treated with different concentrations of SNP for 10 h, whole cell lysates were prepared and subjected to 12% SDS-PAGE gel and Western Blot analysis, detecting phosphorylation of c-Jun. Actin was revealed as a loading control. (E) Cells were treated with different concentrations of SNP for 15 h, and the percentage of dead cells was measured as in (A).

3.3 c-Jun phosphorylation is not dependent on p38 kinase

It has been reported previously that p38 MAP kinase can mediate c-Jun NH_2-terminal phosphorylation (Pramanik *et al*., 2003; Yamagishi *et al*., 2001). Furthermore the activation of p38 MAP kinase occurs in NO donor-induced apoptosis of various neuronal cells (Cheng *et al*., 2001; Ghatan *et al*., 2000). It was important to address the question whether c-Jun phosphorylation is p38-dependent. A p38 specific inhibitor SB203580 was used. SH-Sy5y cells were partially protected from SNP-induced apoptosis by SB203580, suggesting the involvement of p38 in SNP-induced cell death (Fig 3.3A). However, similar extent of c-Jun phosphorylation on Ser-63 was observed with or without SB203580, indicating c-Jun phosphorylation was not dependent on p38 (Fig 3.3B).

3.4 Ser-63 phosphorylation alone mediates NO-induced apoptosis as well as c-Jun and AP-1 transactivation in response to NO in SH-Sy5y cells

To investigate whether c-Jun phosphorylation contributes to NO-induced apoptosis, I stably transfected SH-Sy5y cells with plasmids encoding various dominant-negative forms of c-Jun. In one form, Ser-63 was mutated to alanine (denoted S63A), and in another Ser-73 was mutated to alanine (denoted S73A). In a

third form, both Ser-63 and Ser-73 were mutated to alanines (denoted JunAA), which compromises the ability of c- Jun to transactivate its target genes (Behrens *et al*., 1999).

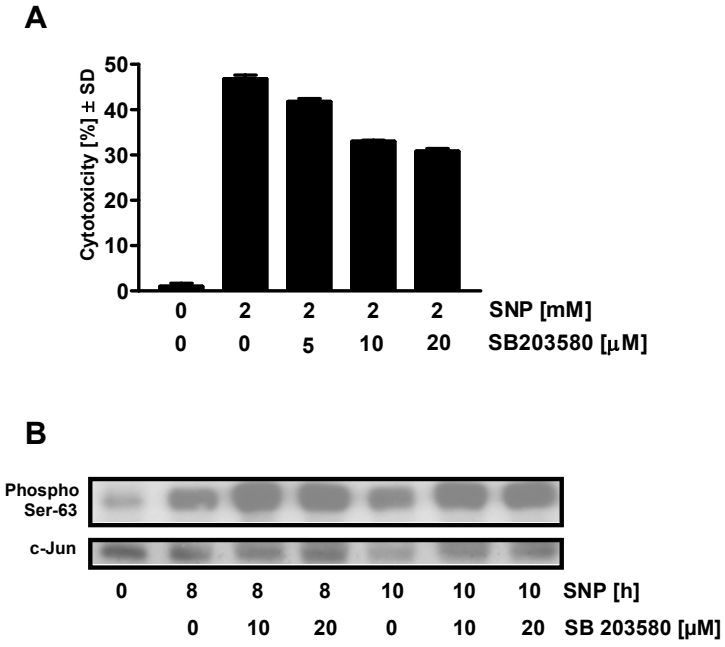

Fig 3.3 c-Jun phosphorylation is not dependent on p38. (A) SH-Sy5y cells were treated with 2 mM SNP with or without the p38 inhibitor, SB203580. 15 h later, the percentage of dead cells was measured by LDH release assay. (B) Cells were treated with 2 mM SNP for different times with or without SB203580. Whole cell lysates were prepared and subjected to 12% SDS-PAGE gel and Western Blot analysis to detect the phosphorylation of c-Jun. c-Jun was revealed as a loading control.

To exclude the possibility that highly overexpressed S63A, S73A, or JunAA might quench JNK activity by sequestering JNK in an abortive complex, I chose for further analysis independent clones in which S63A (Fig 3.4A *upper panel*), S73A (*lower left panel*) or JunAA (Fig 3.4A *lower right panel*) are expressed at levels only slightly in excess of endogenous c-Jun. Normal phosphorylation of endogenous c-Jun on Ser-63 in response to UV irradiation was still observed in two independent clones of S63A stable cells (Fig 3.4B). Analogous results were obtained in UV-irradiation-treated S73A cells (Fig 3.4C). In addition, NO-induced phosphorylation of endogenous c-Jun on Ser-63 still occurred in two independent JunAA clones (Fig 3.4D). These data indicate that the expression of c-Jun mutated to S63A and/or S73A did not compromise endogenous JNK activity.

I then compared the sensitivities of the above three different stable cells and vector control cells to NO donors and UV radiation. At any concentrations of SNP which were sufficient to induce apoptosis, several independent clones of S63A and JunAA stable cells showed markedly increased resistance to cell death compared with vector control cells. Importantly, various S73A stable cell lines failed to show resistance to NO compared with vector control cells (Fig 3.5A). S63A only partially rescued Sy5y cells from NO toxicity, suggesting involvement of other death pathways in addition of phospho-c-Jun. In contrast, neither S63A nor S73A stable cells were resistant to UV irradiation-induced cell death, whereas JunAA cells only showed a marginal increase in resistance to UV irradiation (Fig 3.5B). These data provide evidence that Ser-63 phosphorylation of c-Jun is important in NO-induced, but not UV irradiation-induced cell death.

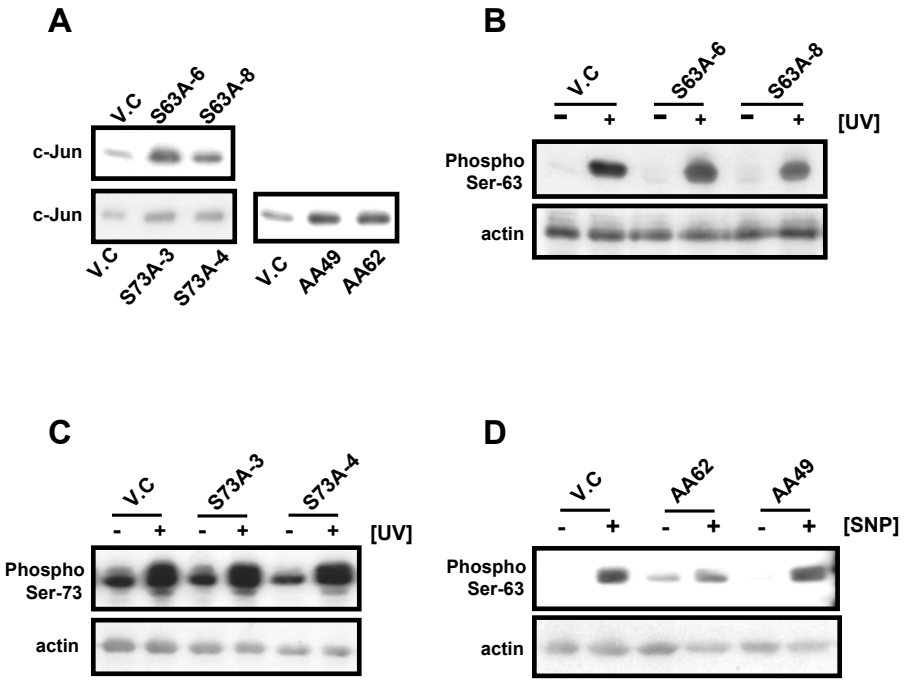

Fig 3.4 Stable expression of S63A, S73 or JunAA does not inhibit endogenous c-Jun phosphorylation in SH-Sy5y cells. (A) Expression levels of the c-Jun proteins in the stable cell lines compared with vector control (*V.C*) cells. *Upper panel*, two clones of S63A. *Lower left panel*, two clones of S73A. *Lower right panel*, two clones of JunAA. Two independent clones of S63A, S73A stable cells and the vector control cells were irradiated by UV at 100 J/m^2 followed by 1 h incubation at 37ºC and whole cell lysates were prepared and subjected to 12% SDS-PAGE gel and Western Blot analysis, detecting the phosphorylation of c-Jun on Ser-63 (B) or Ser-73 (C). Actin was revealed as a loading control. (D) Two independent clones of JunAA stable cells and vector control cells were treated with 2 mM SNP for 10 h and whole cell lysates were prepared. Detection of c-Jun phosphorylation on Ser-63 was done as in (B).

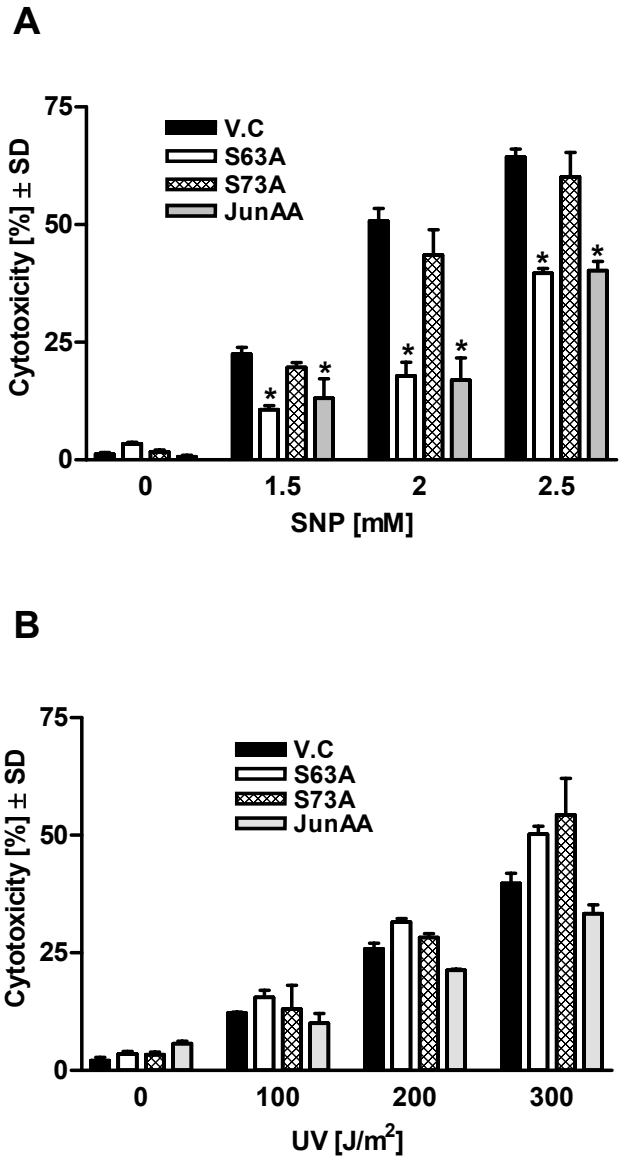

Fig 3.5 Ser-63 phosphorylation alone mediates NO-induced apoptosis in SH-Sy5y cells. S63A, S73A and JunAA stable cells and vector control cells (*V.C*) were treated with different concentrations of SNP for 15 h (A) or different dosages of UV irradiation for 24 h (B), and the percentage of dead cells was measured by LDH release assay. Values are the mean ± S.D. determined from three independent clones of S63A, S73A or JunAA, each in triplicate. Statistical significance of the differences between different cell lines in response to SNP was estimated by Chitest. **P*<0.01 dramatically significant.

Dual phosphorylation of c-Jun on Ser-63 and Ser-73 has been proved previously to lead to c-Jun dependent transactivation (Smeal *et al*., 1991), and accordingly mutation of both serine residues reduces the ability of c-Jun to transactivate target genes (Behrens *et al*., 1999). Because in the current study NO caused c-Jun phosphorylation predominantly on Ser-63, and since S63A and JunAA stable cells showed markedly increased resistance to cell death, the question was addressed whether Ser-63 phosphorylation alone can potentiate c-Jun and AP-1 transactivation. Using Gal4-c-Jun reporter system, I found Gal4-c-Jun (wild type) as well as Gal4-c-Jun (S73A) were transactivated up to 4-fold in SH-Sy5y cells upon NO stimulation (Fig 3.6A). However, Gal4-c-Jun (S63A) and Gal4-c-Jun (JunAA) were completely inactive in transactivation (Fig 3.6A). In parallel experiments, transient transfections with AP-1 reporter plasmids revealed that NO-induced AP-1 activation of up to ~2.7-fold occurred in SH-Sy5y and S73A cells, but was absent in S63A cells (Fig 3.6B). Thus, the combined data from the c-Jun and AP-1 reporter assays indicate that the presence of Ser-63 (but not Ser-73) is required for NO-induced c-Jun/AP-1 transactivation. These results also indicate that the S63A and JunAA constructs function as dominant-negatives by inhibiting gene transcription mediated by endogenous c-Jun.

3.5 Caspase-3 contributes to NO-induced cell death downstream of c-Jun phosphorylation in SH-Sy5y cells

Caspase-3 was found to be important for NO-induced apoptosis in SH-Sy5y cells because prevention of caspase-3 activity by z-DEVD (a selective caspase-3 inhibitor) promoted cell survival (Fig 3.7A). At z-DEVD concentrations that reduce cell death by

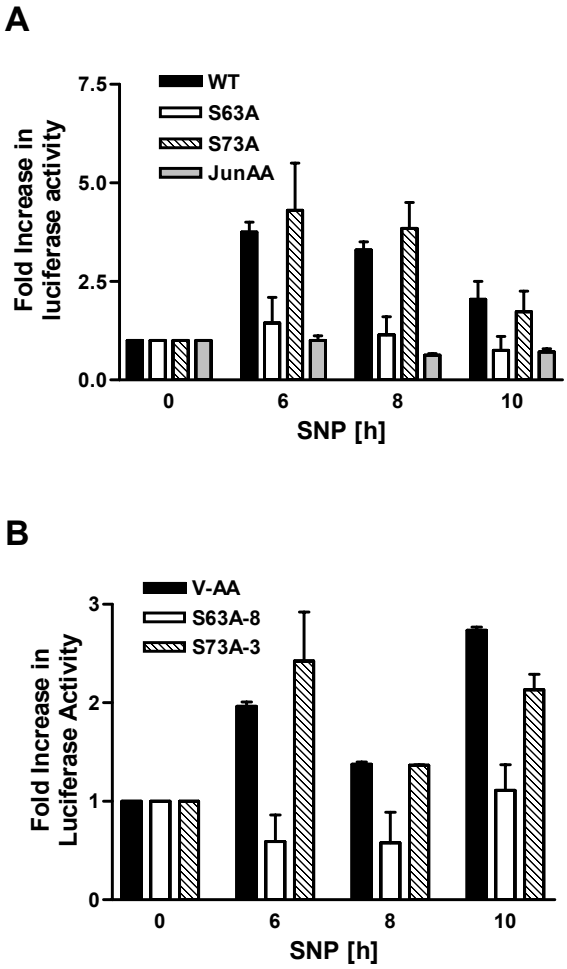

Fig 3.6 Ser-63 phosphorylation is sufficient for c-Jun/AP-1 transactivation in response to NO in SH-Sy5y cells. (A) Gal4-c-Jun reporter assay. Different Gal4-c-Jun constructs bearing the unmodified (*WT*) c-Jun sequence, or S63A, S73A, JunAA mutations in the transactivation region of c-Jun, were cotransfected with a luciferase reporter plasmid together with β-galactosidase into SH-Sy5y cells. 40 h later, the cells were treated with 2 mM SNP for the indicated times and harvested. Luciferase activity was measured, and β-galactosidase activity was also measured as an internal control. (B) AP-1 reporter assay. The TRE-reporter plasmid was co-transfected with the RPL-TK plasmid into S63A, S73A and vector control (*V-AA*) stable cells. 40 h later, the cells were treated with 2 mM SNP for the indicated times and harvested. Firefly luciferase activity was measured, and Renilla luciferase activity was measured as an internal control.

50% (Fig 3.7A) and completely inhibit caspase-3 activity (Fig 3.7B), the levels of NO – induced Ser-63 phosphorylation of c-Jun were similar to those in the absence of z-DEVD (Fig 3.7C), indicating that caspase 3 may act downstream of c-Jun phosphorylation. Two bands of phospho-c-Jun were occasionally observed, (Fig 3.7C) as has been noted previously (Le Niculescu *et al*., 1999b; Leppa *et al*., 1998). I next assayed caspase-3 activity in S63A and S73A stable cells after NO treatment and found that caspase-3 activity was inhibited efficiently in S63A cells compared with vector control cells, whereas S73A cells showed similar (or even slightly enhanced) caspase-3 activity under the same conditions (Fig 3.7D). Since caspase-3 contributes to NO-induced apoptosis in SH-Sy5y cells, these results provide additional evidence that c-Jun phosphorylation on Ser-63 (but not Ser-73) mediates NO-induced apoptosis, and indicate that caspase-3 contributes to apoptosis downstream of c-Jun phosphorylation.

3.6 Evidence that c-Jun phosphorylation in response to NO is directly dependent on JNK in SH-Sy5y cells

To provide more direct proof that JNK(s) play a role in c-Jun phosphorylation in response to NO, I stably transfected SH-Sy5y cells with DN-*jnk1* or DN-*jnk2* plasmids. At least two independent clones of both stable cells exhibited a greatly diminished or absent phosphorylation of c-Jun on Ser-63 (Fig 3.8A and B), indicating that JNKs are directly responsible for NO-stimulated c-Jun phosphorylation in SH-Sy5y cells. Various independently isolated DN-*jnk1* and DN-*jnk2* clones also showed increased resistance to apoptosis at three concentrations of NO donor (Fig 3.8C), that was quantitatively similar to that observed in S63A cells. A marked decrease of caspase-3 activity, indicative of increased survival, was also observed in these stable cells. This combined evidence suggests that inhibition of JNK leads to enhanced cell

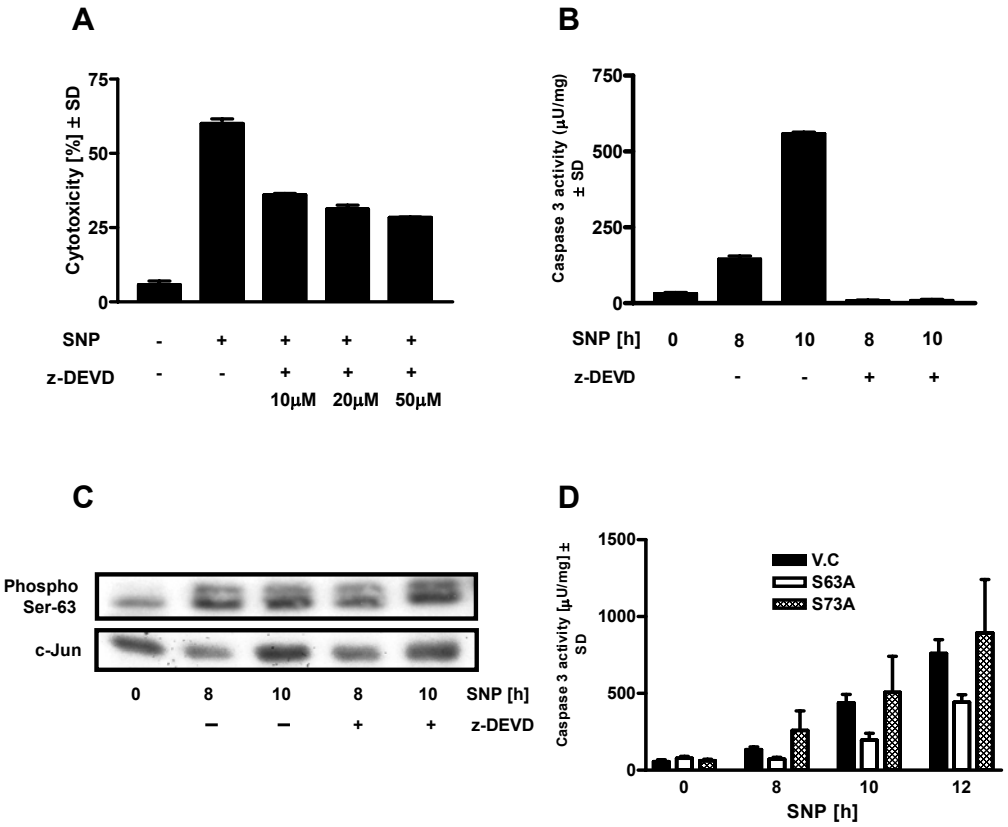

Fig 3.7 Caspase-3 contributes to NO-induced cell death downstream of c-Jun phosphorylation in SH-Sy5y cells. (A) SH-Sy5y cells were treated with 2 mM SNP in the absence or presence of the indicated concentrations of caspase-3 inhibitor z-DEVD for 15 h. Percentage of dead cells was measured by LDH release assay. (B) SH-Sy5y cells were treated with 2 mM SNP in the presence of 20 μM z-DEVD for the indicated times, and caspase-3 activity was measured. In A and B, values are the mean ± S.D. determinded from three experiments performed in triplicate. (C) Cells were treated with 2 mM SNP in the absence or presence of z-DEVD for the indicated times. Whole cell lysates were prepared and subjected to SDS-PAGE gel and Western Blot analysis, detecting phosphorylation of c-Jun. C-Jun was revealed as a loading control. (D) S63A, S73A and vector control (*V.C*) cells were treated with 2 mM SNP for the indicated times, and the caspase-3 activity was measured as described in "materials and methods". Values are the mean ± S.D. determined from three independent clones of S63A, S73A, each performed in triplicate.

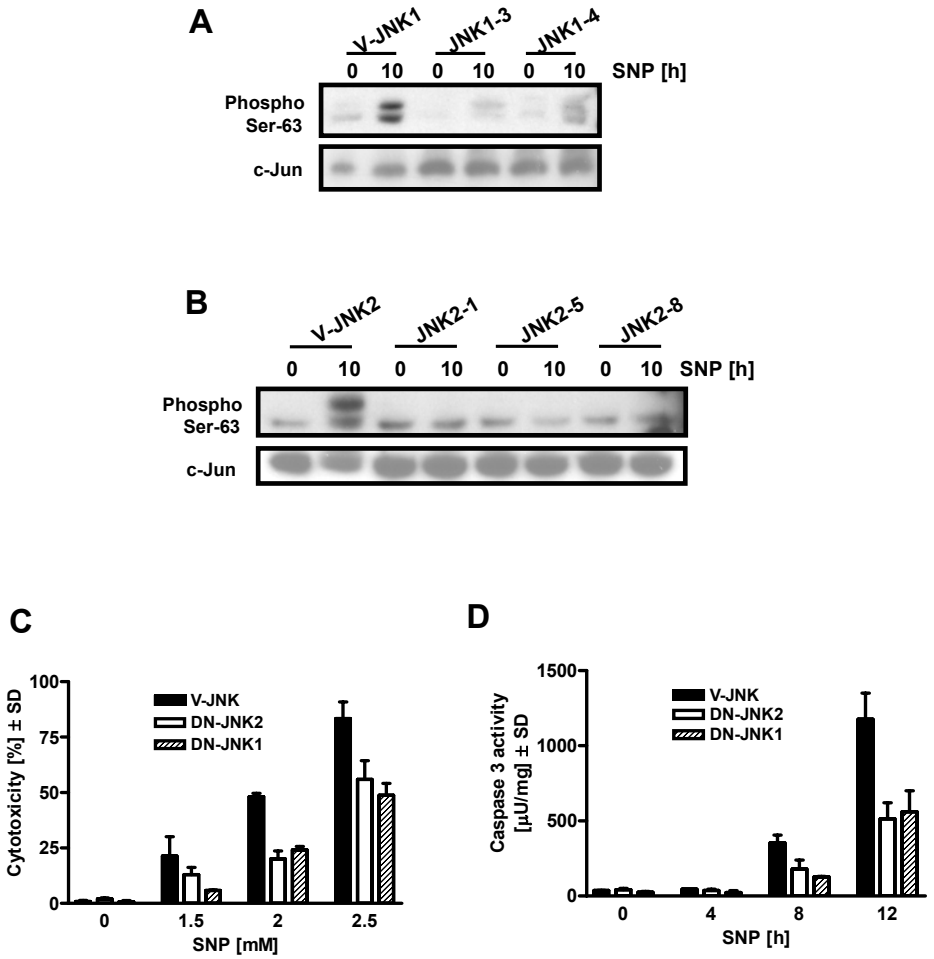

Fig 3.8 Evidence that c-Jun phosphorylation in response to NO is directly dependent on JNK in SH-Sy5y cells. DN-*jnk1* (A) or DN-*jnk2* (B) stable cells and vector control (*V-JNK*) cells were exposed to 2 mM SNP for up to 10 h. Whole cell lysates were prepared and subjected to SDS-PAGE gel and Western Blot analysis, detecting the phosphorylation of c-Jun. (C) DN-*jnk1* or DN-*jnk2* stable cells and the vector control cells (*V-JNK*) were exposed to different concentrations of SNP for 15 h. The percentage of dead cells was measured by LDH release assay. (D) DN-jnk1 or DN-jnk2 stable cells and the vector control cells (*V-JNK*) were exposed to 2 mM SNP for different times, and caspase-3 activity was measured. In both (C) and (D), values are the mean ± S.D. determined from three independent clones of DN-*jnk1* or DN-*jnk2* cells, each in triplicate.

survival through the suppression of JNK-dependent Ser-63 phosphorylation of c-Jun and the inhibition of caspase-3 (Fig 3.8D).

3.7 Evidence that JNK-mediated c-Jun phosphorylation on Ser-63 is a general phenomenon in NO-induced apoptosis of neuroblastoma cells

It was important to find out whether JNK-mediated c-Jun phosphorylation on Ser-63 plays a general role in NO-induced apoptosis in neuroblastoma cell lines and to employ an alternative strategy to block JNK activity. In SHEP neuroblastoma cells, a strong Ser-63 phosphorylation of c-Jun began at around 8 h after SNP treatment, whereas Ser-73 phosphorylation was again virtually undetectable (Fig 3.9A). As with SH-Sy5y cells, UV irradiation caused similar extent of phosphorylation of both Ser-63 and Ser-73 residues in SHEP cells (Fig 3.9A). A cell-permeable peptide, D-JNKI1 (JNK inhibitory peptide 1), which specifically inhibits JNK activity (Bonny *et al*., 2001), effectively blocked both Ser-63 phosphorylation (Fig 3.9B) and death of SHEP cells (Fig 3.9C). In contrast, a control cell-permeable peptide, D-TAT, neither prevented Ser-63 phosphorylation nor the death of SHEP cells (Fig 3.9B and 3.9C, respectively). These results offer additional evidence that the JNK family of protein kinases phosphorylate c-Jun in NO-induced apoptosis and argue that JNK-mediated c-Jun phosphorylation on Ser-63 plays an important role in triggering the death of neuroblastoma cells.

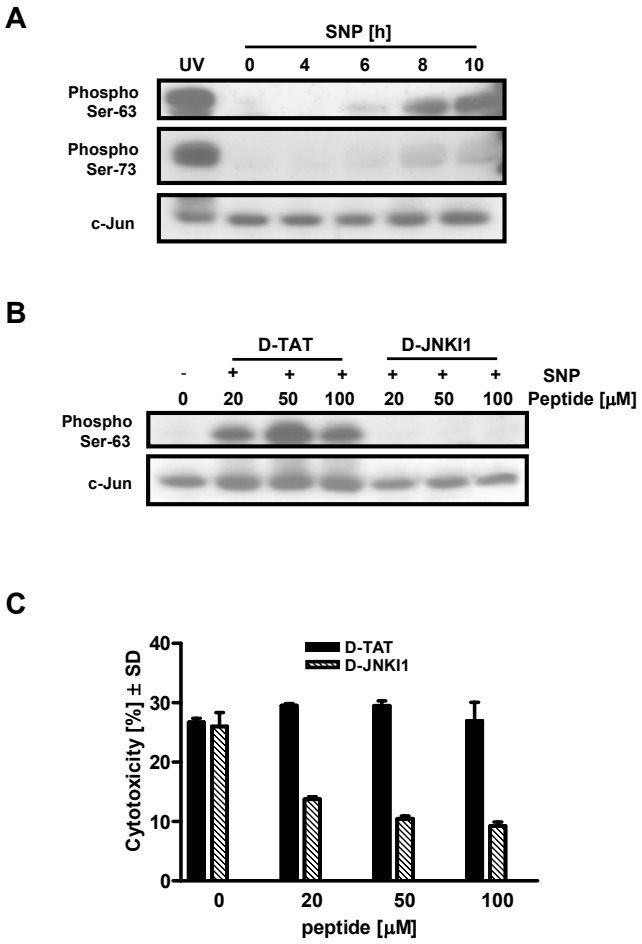

Fig 3.9 Evidence that JNK-mediated c-Jun phosphorylation on Ser-63 is a general phenomenon in NO-induced apoptosis of neuroblastoma cells. (A) SHEP cells were treated with 100 J/m^2 followed by 1 h incubation at 37ºC or 2 mM SNP for the indicated times and lysed. Whole cell lysates were subjected to SDS-PAGE gel and Western Blot analysis, detecting the phosphorylation of c-Jun on Ser-63 or Ser-73. SHEP cells treated with UV (100 J/m^2) acted as a positive control for dual Ser-63 and Ser-73 phosphorylation. c-Jun was revealed as a loading control. (B) SHEP cells were treated with 2 mM SNP plus different indicated concentrations of a cell-permeable JNK inhibitory peptide (D-JNKI1) or control peptide (D-TAT) for 10 h. c-Jun phosphorylation on Ser-63 was evaluated as described in A. (C) SHEP cells were treated with 2 mM SNP plus different indicated concentrations of D-JNKI1 or D-TAT peptides for 15 h, and the percentage of cell death was measured by LDH release assay. Values are the mean ± S.D. determined from experiments performed in triplicate.

3.8 Discussion

Previous reports have indicated that excessive generation of NO might be coupled to the activation of signal transduction cascades involving stressed-activated protein kinases and transcription factors. The activation of p38 MAP kinase occurs in NO-donor induced apoptosis of various neuronal cells (Cheng *et al*., 2001; Ghatan *et al*., 2000), which in one case involved p38 acting upstream of Bax to trigger the intrinsic (mitochondria) death pathway (Ghatan *et al*., 2000). There are many reports that NO can regulate AP-1 in the brain (Bogdan, 2001; Kataoka and Yanase, 1998), and various other studies have demonstrated that c-Jun/AP-1 can modulate apoptosis induced by diverse agents (Estus *et al*., 1994; Kihiko *et al*., 1999; Leppa *et al*., 2001; Potapova *et al*., 2001; von Knethen *et al*., 1999). However, the existence of NO-induced JNK-c-Jun signaling and subsequent gene regulation in apoptosis has not been explored until now. In neurons, the JNK-c-Jun pathway is pro-apoptotic during neurotrophin factor withdrawal, kainate treatment, and potassium deprivation. This involves Ser-63 and Ser-73 phosphorylation (Behrens *et al*., 1999; Harada and Sugimoto, 1999; Le Niculescu *et al*., 1999), and it is accepted that the transcriptional activation of c-Jun in cell growth and development depends strictly on the dual phosphorylation of these amino acids (Behrens *et al*., 1999; Smeal *et al*., 1991).

It is, therefore, notable I now show that Ser-63 (but not Ser-73) phosphorylation of c-Jun mediates NO-induced apoptosis of neuroblastoma cells. The evidence came from several complementary lines of investigation. Firstly, NO induced a strong activation of JNK, and only toxic concentrations of SNP induced phosphorylation of c-Jun on Ser-63 prior to and at the onset of apoptosis. Secondly, S63A and JunAA stable cells (but not S73A cells) exhibited significantly increased resistance to apoptosis triggered by NO as measured by cell death and caspase-3 assays. Moreover, DN-*jnk1*

and DN-*jnk2* stable cells showed increased resistance to NO correlating with markedly reduced Ser-63 phosphorylation and caspase-3 activation. My data suggest that JNKs are primarily responsible for phosphorylating c-Jun on Ser-63, which is further supported by my unpublished observation that a specific p38 MAP kinase inhibitor failed to block Ser-63 phosphorylation of c-Jun. Third, in a different approach, a highly specific JNK-inhibitory peptide blocked both exclusive Ser-63 phosphorylation and NO-induced apoptosis of SHEP neuroblastoma cells.

Might one or both of the JNKs (1 and 2) directly phosphorylate substrates other than c-Jun and thereby contribute to apoptosis? Although still controversial, there is evidence that JNK-mediated phosphorylation of p53, $p66^{shcA}$ or Bcl-2 family members is pro-apoptotic in various different contexts (Buschmann *et al*., 2001; Fan *et al*., 2000; Kharbanda *et al*., 2000; Le *et al*., 2001; Yamamoto *et al*., 1999). However, I showed here that JNK activity is present at normal levels in S63A, JunAA and S73A cells, arguing that alternative potential JNK death substrates other than c-Jun would still be phosphorylated under conditions in which S63A protects from NO killing. Thus Ser-63 of c-Jun is the important target in the NO-inducible killing pathway. It is not known why NO induces only Ser-63 phosphorylation, but it is worth speculating. There are 10 known isoforms of JNKs, and NO might activate one isoform that only targets Ser-63. Alternatively, NO might activate a Ser-73 phosphatase; or putative NO-mediated chemical modification (e.g. nitrosylation) of c-Jun could preferentially block Ser-73 phosphorylation.

What is the pathway by which Ser-63 phosphorylation of c-Jun connects to caspase-3 activation and mediates apoptosis? There are two major possibilities. First, Ser-63 phosphorylation might activate c-Jun, which then transactivates death genes (or suppresses protective genes). This is strongly supported by the results of the Gal4-c-

Jun and AP-1 reporter assays which show that the S63A mutation alone abolishes transactivation. These assays also indicate that c-Jun is a crucial component of an AP-1 complex activated by NO, in agreement with antibody supershift experiments demonstrating c-Jun protein is abundant in AP-1 complexes after NO stimulation (Feng *et al*., 2002). Speculatively, Ser-63 phosphorylation might regulate a different set of target genes compared with dual phosphorylated or non-phosphorylated c-Jun, resulting in a shift to pro-cell death gene expression. It is worth considering the known c-Jun-regulated genes that are thought to play roles in neuronal apoptosis (Mattson *et al*., 2000; Pennypacker, 1997; Pennypacker, 1995; Shaulian and Karin, 2001; Shaulian and Karin, 2002; Tong *et al*., 1998). Among the stronger candidates are *hrk*, *bim* and *fasl*, which are pro-apoptotic (Harris and Johnson, Jr., 2001; Kasibhatla *et al*., 1998; Kolbus *et al*., 2000; Le Niculescu *et al*., 1999; Morishima *et al*., 2001; Whitfield *et al*., 2001), but other possible genes are *bcl-3* and *GAP43* (Haas *et al*., 2000; Rebollo *et al*., 2000). The second, perhaps less likely, possibility is Ser-63 phosphorylation may lead to apoptosis through transcriptional repression due to actions of c-Jun that suppress or antagonize other transcription factors (Herdegen and Waetzig, 2001).

Recently, single phosphorylation of one of the two serine residues of c-Jun N-terminal domain has been published (Yu *et al*., 2003). In cultured sympathetic neurons both deprivation of nerve growth factor (NGF) and deprivation of glial cell line–derived neurotrophic factor (GDNF) results in massive cell death. But the underlying mechanisms are different. Serine 73 of c-Jun was phosphorylated in both NGF- and GDNF-deprived neurons, whereas serine 63 was phosphorylated only in NGF-deprived neurons. Although there was no extensive study on the biological significance of Ser73 phosphorylation of c-Jun in GDNF withdrawal induced cell death, it did provide additional evidence that under certain circumstances, single phosphorylation of c-Jun

can occur, and may have important physiological relevance. Furthermore, the important role of serine 63 phosphorylation in the cellular response to oxidative stress has been proposed (Madsen *et al*., 2004). Long-lived Snell Dwarf mice respond to oxidative stress induced by 3-NPA differently compared to their wild type littlemates (3-NPA is an irreversible inhibitor of succinate dehydrogenase, a component of mitochondira complex II, that generates free radicals). A significantly lower response in the MEK-ERK kinase cascade and a complete lack of c-Jun phosphorylation on serine 63 were observed in Snell Dwarf mice but not in wild type mice, suggesting a role of serine 63 in mediating an oxidative stress response. This altered management of oxidative stress may also provide an explanation for the extended longevity in dwarf mice, due to the role of oxidative stress in aging (Migliaccio *et al*., 1999; Sampayo *et al*., 2003).

CHAPTER 4 *sgII*, an AP-1 target gene, is a new class of proteins that mediate neuroprotection from NO-induced apoptosis and NGF-induced neuronal differentiation in SH-Sy5y cells

AP-1 transcription factors can regulate cell viability in culture, showing high complexity and even apparent contradictions depending on the cell type analyzed and stimuli exerted on cells. The diverse effects of AP-1 on cell death and survival probably arise from the diversity of AP-1 dimers as well as activation of different groups of pro- or anti-apoptotic target genes (Herdegen *et al*., 1997; Karin *et al*., 1997; Shaulian and Karin, 2002; Wisdom, 1999). However, the molecular targets of AP-1 in cell death and survival are incompletely understood. Notably, AP-1 activity is enhanced in the CNS under pathological conditions such as acute or chronic neuronal disorders, suggesting a functional correlation of AP-1 and pathophysiology in these scenari. But downstream events, in terms of AP-1 target genes, are still unclear.

Secretogranin II (SgII), an abundant component of neuroendocrine granules, might be regulated by AP-1 because of a conserved cyclic AMP response element (CRE) motif in its promoter that is important for both basal and regulated transcription of *sgII* (Desmoucelles *et al*., 1999; Mahata *et al*., 1999; Mahata *et al*., 2002; Scammell *et al*., 2000). Besides its classical function with relevance to secretion, there is growing evidence that SgII also involves in other cellular events such as modulation of neurotransmission, inflammatory responses and neuronal differentiation (Fischer-Colbrie *et al*., 1995; Taupenot *et al*., 2003).

4.1 Dominant-negative c-Jun (TAM-67) sensitizes SH-Sy5y cells to NO-induced apoptosis

From my previous study, it is known that Ser-63 phosphorylation of c-Jun mediates NO-induced cell death in neuroblastoma cells. Since phosphorylation of Ser-63 and Ser-73 are dispensable for c-Jun activity, it is worth of looking into the global role of c-Jun/AP-1 in NO-induced cell death. To address this question, another dominant-negative form of c-Jun, TAM-67, was adopted. TAM-67 lacks most of the transactivation domain (TAD) of c-Jun and is able to inhibit the global function of endogenous c-Jun and AP-1through a competitive mechanism (Brown *et al*., 1994).

Since components of AP-1 dimers can vary under different circumstances, it is important to know whether c-Jun is part of the AP-1 complex stimulated by NO. Besides usage of a c-Jun reporter assay (described in section 3.4), another approach, EMSA and supershift, was used. NO was able to increase AP-1 affinity to its consensus binding motif in a time-dependent style in an *in vitro* binding experiments as described in the "materials and methods", starting from 2 h and reaching its peak about 8 h after SNP addition (Fig 4.2, *left panel*). To determine whether c-Jun is present in this AP-1 complex, nuclear extracts from the 8 h time point were incubated with the AP-1-specific oligonucleotide with or without antibody against c-Jun. As shown in Fig 4.2, *right panel*, AP-1 binding was completely blocked in the presence of c-Jun antibody, suggesting that c-Jun is a crucial component of the AP-1 complex induced by SNP. Dr. Zhiwei Feng contributed the supershift data (Feng *et al*., 2002).

To clarify the global function of AP-1 in NO-induced apoptosis, Dr. Zhiwei Feng generated stable SH-Sy5y cell line expressing TAM-67 (Fig 4.3A). As mentioned earlier, TAM-67 has 104 amino acids deleted from the TAD of c-Jun and is able to dimerize efficiently with Fos, Jun and ATF-2 family proteins and bind the AP-1

consensus motif. Theoretically it can quench or inhibit endogenous AP-1 activity as illustrated in Fig 4.1.

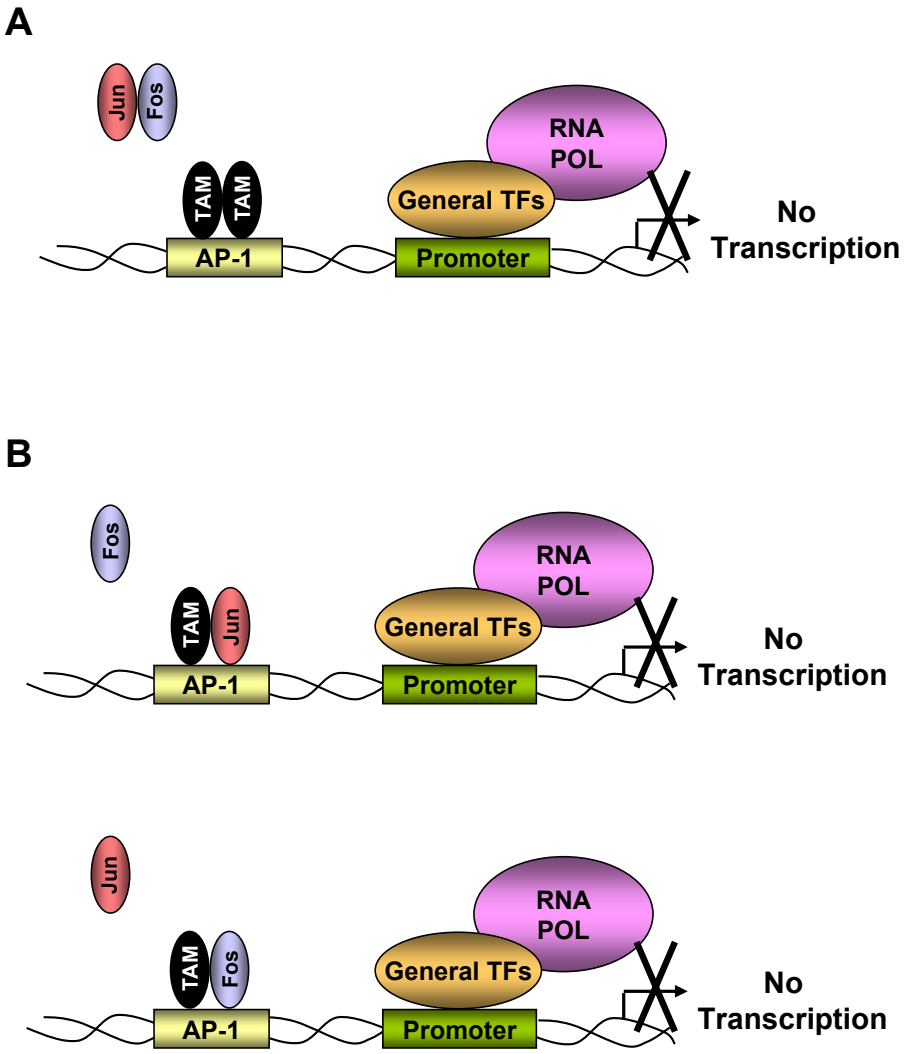

Fig 4.1 Inhibition of TAM-67 on endogenous AP-1 through competitive mechanisms. (A) TAM-67 is able to dimerize with itself, occupies the AP-1 binding sites on the target gene promoter region and thus "kick the endogenous AP-1 out". This is called a "blocking mechanism". (B) TAM-67 is also able to dimerize with AP-1 subfamily proteins such as Jun or Fos, which then occupy the AP-1 binding sites on the target gene promoter region and thus prevent functional dimerization between wild type AP-1 components. This is called a "quenching mechanism" (Brown *et al*., 1994).

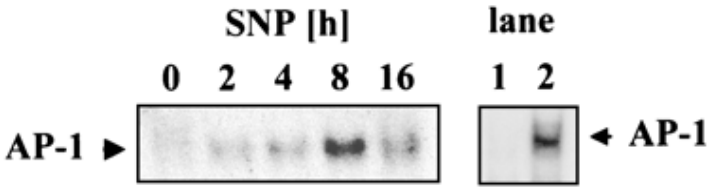

Fig 4.2 NO stimulates AP-1 activity in SH-Sy5y cells, and c-Jun is the major component of the AP-1 complex. DNA bandshift analyses in native polyacrylamide gels were performed using an oligonucleotide containing consensus AP-1 motif. *Left panel*, nuclear extracts of SH-Sy5y cells were prepared at the indicated times after 2 mM SNP treatment. *Right panel*, supershift analyses using c-Jun antibodies. Nuclear extracts were prepared from SH-Sy5y cells treated with 2mM SNP for 8 h. Lane 1, 1 μg of polyclonal antibody against c-Jun was added before the AP-1 oligonucleotide. Lane 2, no c-Jun antibody was added (Feng *et al*., 2002).

To establish that TAM-67 is functional when expressed in SH-Sy5y cells, I compared the endogenous AP-1 activity in vector control cells and TAM-67 stable cells. AP-1 recognizes two types of consensus motif defined as TRE (TPA-responsive element, 5' TGAC/GTCA3') and CRE (cAMP-responsive element, 5'TGACGTCA3'). Reporter plasmids bearing either TRE or CRE as enhancer element for luciferase gene expression were transfected into vector control cells and TAM-67 stable cells, and luciferase activity was measured. As shown in Fig 4.3B, TAM-67 overexpression caused a dramatic inhibition of both TRE- and CRE-mediated reporter gene expressions. These data suggest that TAM-67 expression causes a global blockage of AP-1 mediated transcription in SH-Sy5y cells. Utilizing TPA (a classical AP-1 activator), I also found that besides basal AP-1 activity, inducible AP-1 activity was also greatly inhibited in TAM-67 cells compared with vector control cells (Fig 4.3C).

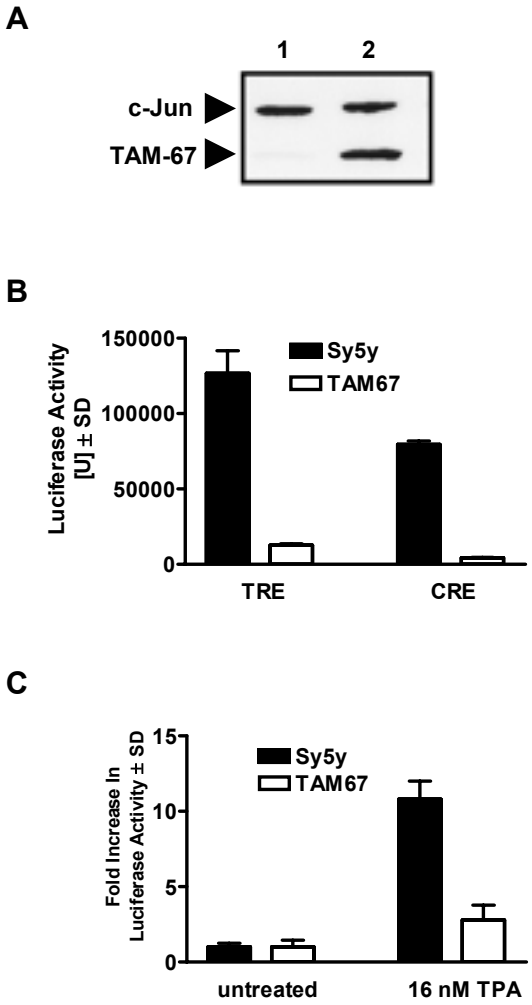

Fig 4.3 TAM-67 stable expression in SH-Sy5y cells blocks the endogenous AP-1 activity. (A) Western blot analysis of SH-Sy5y cells stably expressing TAM-67 constructs. Lane 1, vector control cells. Lane 2, cells overexpressing TAM-67 (Feng *et al*., 2002). The positions of parental c-Jun and TAM-67 (a truncated form of c-Jun) are shown with an arrowhead. (B) Endogenous AP-1 activity in parental and TAM-67 stable SH-Sy5y cells. CRE- or TRE-reporter plasmids were co-transfected with RPL-TK plasmids into two different cell lines. 40 h later, cells were harvested and firefly luciferase activity was measured. Renilla luciferase activity was measured as an internal control. (C) TRE-reporter plasmids were co-transfected with RPL-TK plasmids into two cell lines. 40 h later, cells were treated with 16 nM TPA for 8 h and luciferase activity was measured as in (B).

Since AP-1 was shown to play a role in apoptosis under different circumstances, it was important to elucidate the function of AP-1 in NO-induced apoptosis in SH-Sy5y cells. The sensitivity of vector control cells and TAM-67 stable cells to NO was compared. TAM-67 stable cells were markedly more sensitive to SNP-induced apoptosis than vector control cells at different concentrations (Fig 4.4A). To independently verify the difference in sensitivities of vector control and TAM-67 stable cells to SNP, the externalization of phosphatidylinositol was quantitated by flow-cytometry-based annexin V staining. In most normal, viable eukaryotic cells, the negatively charged phospholipid phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer. PS redistribution from the inner to the outer leaflet is an early and widespread event during apoptosis. Annexin V is a 35 kDa phospholipid-binding protein and has a high affinity to PS in the presence of physiological concentrations of calcium (Ca^{2+}). In contrast to vector control cells, there was appreciable annexin V staining of TAM-67 cells, which again shows that suppression of AP-1 activity sensitizes SH-Sy5y cells to cell death induced by SNP. Similar results were obtained with an alternative NO donor, SIN-1, which also kills SH-Sy5y cells by apoptosis. Figure 4.C shows that the SIN-1-induced death of TAM-67 cells was markedly higher than that of vector control cells (data was contributed by Dr Zhiwei Feng), confirming that blocking of AP-1 function sensitizes SH-Sy5y cells to NO toxicity.

4.2 Protective gene expression is partially responsible for counteracting NO-toxicity

It is well established that NO regulates gene expression (Bogdan, 2001), but it is unclear how this might influence the susceptibility of neuronal cells to NO-induced

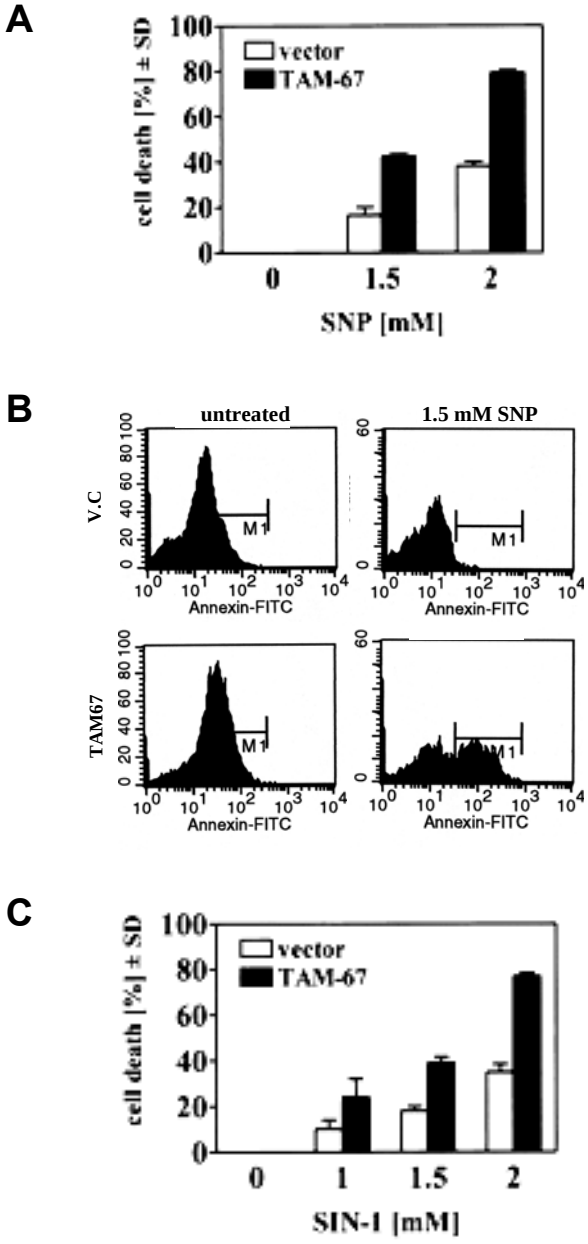

Fig 4.4 TAM-67 over-expression sensitizes SH-Sy5y cells to NO toxicity. (A) Vector control cells and TAM-67 cells were treated with the indicated concentrations of SNP for 16 h, and the percentage of cell death was quantitated using crystal violet staining as described in the "materials and methods". Values were the mean and S.D was from three experiments performed in triplicate. (B) Cell death was determined by flow cytometry using annexin V staining to detect externalized phosphatidylserine (PS). Vector control cells and TAM-67 cells were left untreated or treated with 1.5 mM SNP for 16 h. M1 marks the positions of the annexin V-stained cells. (C) Vector control cells and TAM-67 stable cells were treated with the indicated concentrations of SIN-1 for 16 h, and the percentage of cell death was quantitated using crystal violet staining (Feng *et al*., 2002).

apoptosis. Inhibition of transcription or translation in SH-Sy5y cells by nontoxic doses of actinomycin D (AD) or cycloheximide (CHX) sensitized the cells to NO-induced cell death (Fig 4.5), suggesting that new or continuous gene expression is required for protection of these cells from NO toxicity.

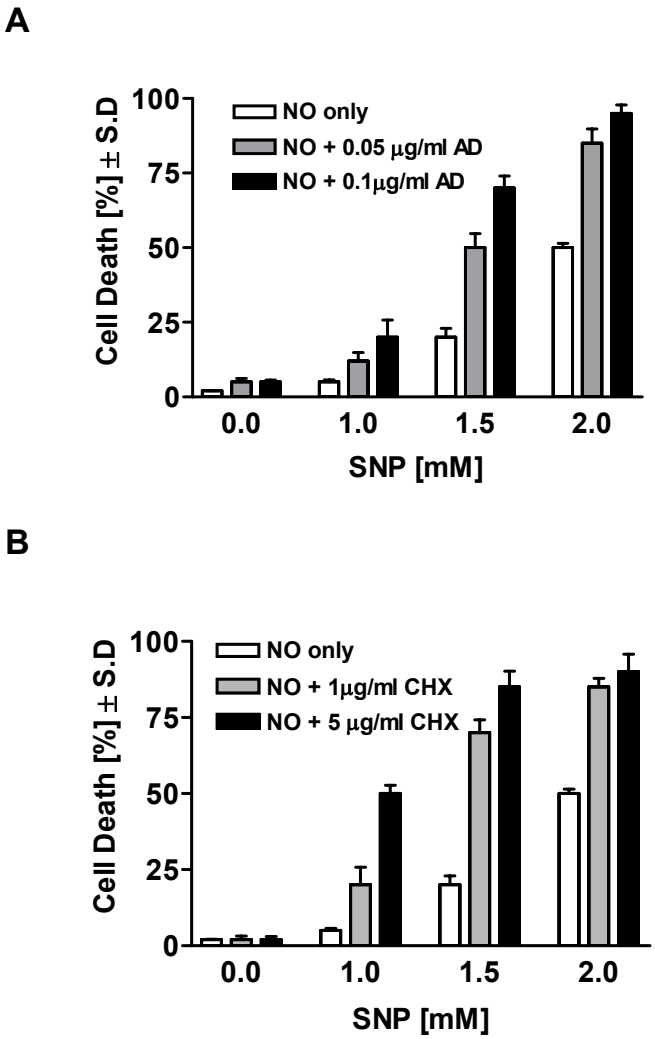

Fig 4.5 Putative protective genes counteract NO toxicity in SH-Sy5y cells. SH-Sy5y cells were treated with SNP alone or together with two different concentrations of actinomycin D (AD) or cycloheximide (CHX) for 16 h. The percentage of cell death was measured as in Fig 4.4 by crystal violet staining. Values were the mean and the S.D was from three experiments performed in triplicate.

4.3 *sgII*, a potential AP-1 target gene, shows an NO-inducible and AP-1-dependent expression pattern in SH-Sy5y cells

Based on the observations described above, TAM-67 overexpression leads to dramatic sensitization of SH-Sy5y cells to NO-induced cell death, and inhibition of transcription as well as translation could render the cells more sensitivity to NO. Furthermore, since TAM67 overexpression caused a massive blockade of AP-1 mediated transcription in SH-Sy5y cells, I hypothesized that some neuroprotective gene(s), whose expression is AP-1 dependent, could be involved in counteracting NO killing. To identify these gene(s), a microarray analysis was carried out, comparing the gene expression profiles in 6hr or 10hr SNP-treated SH-Sy5y cells and TAM67 stable cells. At 6 and 10h after SNP treatment, *sgII* mRNA was respectively 5.2 and 17.7-fold lower in the TAM67 cells compared to the SH-Sy5y cells (Fig 4.6A), suggesting that c-Jun/AP-1 may be involved in the NO-induced transcriptional induction of the *sgII* gene.

Semi-quantitative RT-PCR confirmed and extended the microarray results, showing that basal and NO-inducible *sgII* mRNA expression was markedly reduced in TAM67 cells compared to SH-Sy5y cells treated for various times with either NO donor, SNP or DETA-NO (Fig 4.6B). Since NO stimulated CRE activity in SH-Sy5y cells (Fig 4.7A), and a CRE element in the human *sgII* promoter region is important for tissue-specific and inducible expression of the gene (Desmoucelles *et al*., 1999; Mahata *et al*., 2002), I addressed the question whether the CRE located within the *sgII* promoter is sufficient to mediate NO-induced transcriptional up-regulation of *sgII*. A short DNA region with the *sgII* promoter and its CRE element was placed upstream of a luciferase reporter. NO activated substantial luciferase activity when this construct

A

SNP [h]	Sy5y	TAM67	Fold difference in expression
6	1533	297	5.2
10	7923	447	17.7

B

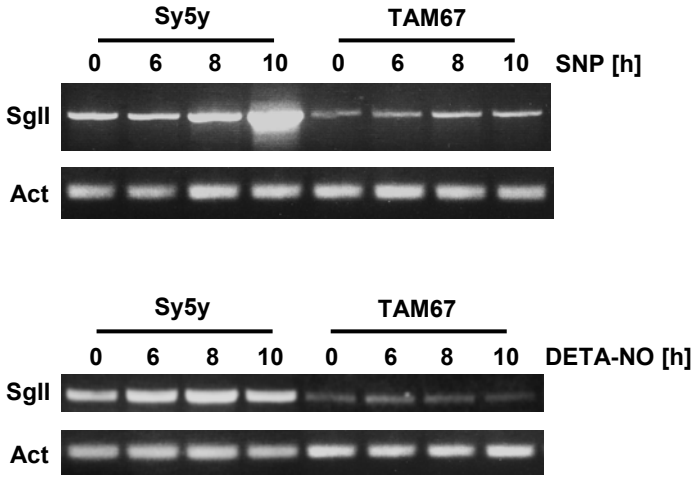

Fig 4.6 *sgII* expression is mediated by c-Jun and is NO-inducible in SH-Sy5y cells. (A) Microarray data comparing *sgII* expression in SH-Sy5y and TAM67 stable cells at two different time points following 2 mM SNP treatment. The numbers in the second and third columns refer to the absolute values of cy3 and cy5 fluorescence from which the fold reduction in sgII expression was calculated (column 4). (B) SH-Sy5y cells and TAM67 cells were treated with 2 mM SNP or DETA-NO for the indicated times, total RNA was extracted and subjected to RT-PCR to detect *sgII* transcripts. Act, actin.

was transfected in SH-Sy5y cells, as did TPA acting as a positive control (Fig 4.7B). However, neither NO nor TPA activated luciferase activity when the same construct was transfected in TAM67 cells, and the basal luciferase activity was much lower in unstimulated TAM67 cells than in SH-Sy5y cells (Fig 4.7B), suggesting AP-1 is important for the expression of *sgII* in SH-Sy5y cells. To gain more direct evidence that CRE is important for *sgII* transcription, the CRE core box of the *sgII* promoter was mutated (Andrecht *et al*., 2002) and the reporter analysis was repeated. As shown in Fig 4.7C, basal, NO-inducible and TPA-inducible luciferase expression were all virtually abolished, indicating the critical importance of the CRE element in mediating *sgII* expression.

Since the genes encoding other chromogranin family proteins (CgA, CgB and *sgV*) share some common cis-regulatory elements in their promoters (Taupenot *et al*., 2003), I also examined the expression patterns of their mRNAs. Although, *cgA*, *cgB* and *sgV* each exhibited a basal level of expression in SH-Sy5y cells, none showed NO-induced up-regulation like *sgII* (Fig. 4.8A); but strikingly, the expression of *sgV* was totally dependent on c-Jun/AP-1 (Fig. 4.8B). Altogether, these data show that *sgII* and *sgV* are regulated in large part by c-Jun/AP-1, and suggest that the single CRE element located upstream of the *sgII* gene is involved in c-Jun/AP-1-dependent basal expression and NO-stimulated induction of *sgII*.

Consistent with the RT-PCR analyses, SgII protein was also considerably reduced in c-Jun-inhibited TAM67 cells compared to parental SH-Sy5y cells (Fig 4.9A). Unlike the NO-inducible upregulation of *sgII* transcripts, the intracellular SgII protein remained at a constant level following NO treatment (Fig 4.9B, *top panel*). The apparent failure to detect a corresponding up-regulation of SgII protein may be partly

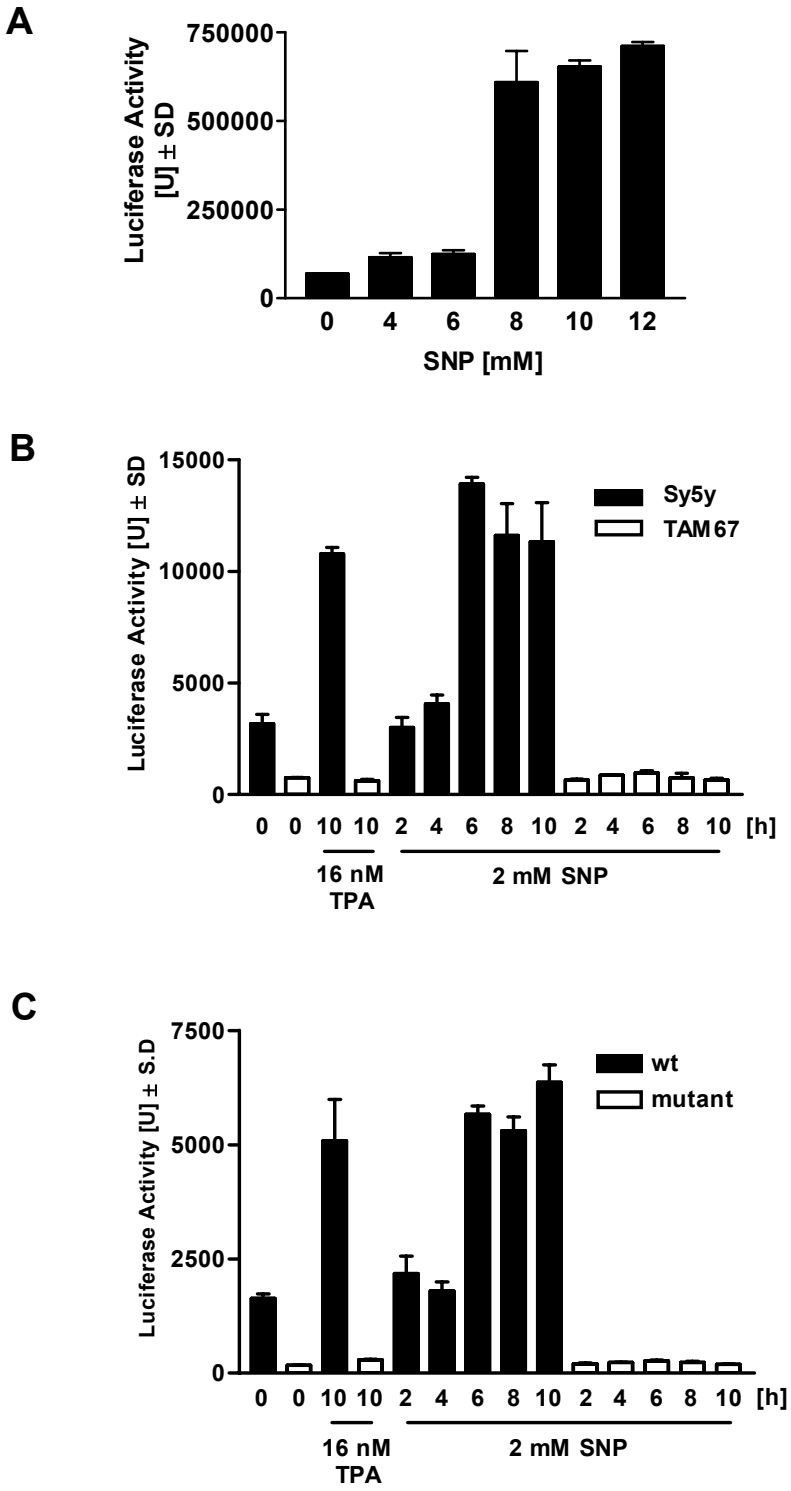

Fig 4.7 *sgII* expression is mediated by c-Jun/AP-1 and requires a CRE motif in the sgII promoter. (A) SH-Sy5y cells were transfected with a CRE-luciferase reporter plasmid. 40 h after transfection, cells were treated with 2 mM SNP for the indicated times, harvested and luciferase activity was measured. (B) SH-Sy5y cells (filled bars) or TAM67 cells (open bars) were transfected with a luciferase reporter plasmid bearing the human *sgII* promoter sequence. 40 h after transfection, cells were treated with 16 nM TPA or 2 mM SNP for the indicated times and harvested. Luciferase activity was measured. (C) SH-

Sy5y cells were transfected with a luciferase reporter plasmid bearing either the wild type *sgII* CRE (filled bars) or a mutated CRE (open bars). 40 hr after transfection, cells were treated with 16 nM TPA or 2 mM SNP for the indicated times and harvested. Luciferase activity was measured.

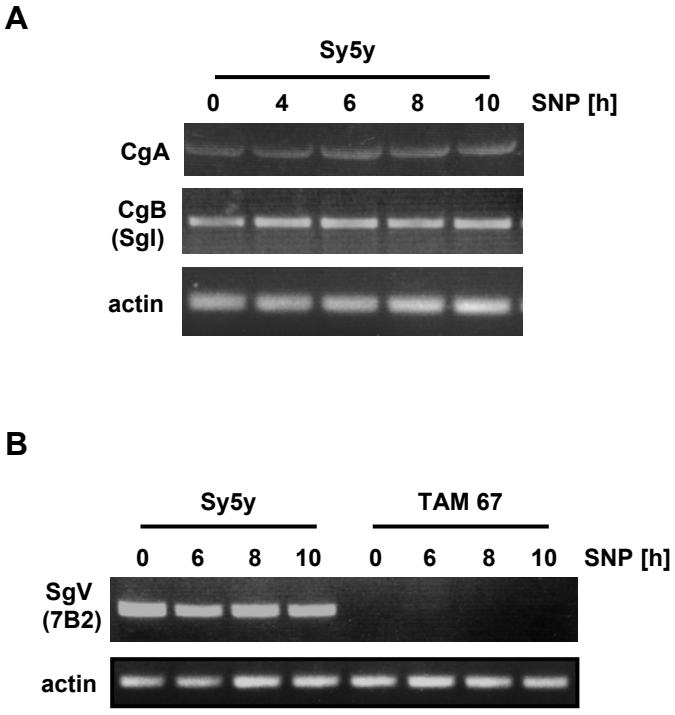

Fig 4.8 Expression patterns of various chromogranin genes. (A) NO-inducible mRNA expression patterns of members of the chromogranin family in SH-Sy5y cells. SH-Sy5y cells were treated with 2 mM SNP for the indicated times, total RNA was extracted and subjected to RT-PCR to detect the transcripts of *cgA* and *cgB* by agarose gel electrophoresis. (B) Basal and NO-inducible mRNA expression patterns of *sgV* in SH-Sy5y cells and TAM67 cells. SH-Sy5y cells and TAM67 cells were treated with 2 mM SNP for the indicated times, total RNA was extracted and subjected to RT-PCR to detect *sgV* transcripts by agarose gel electrophoresis.

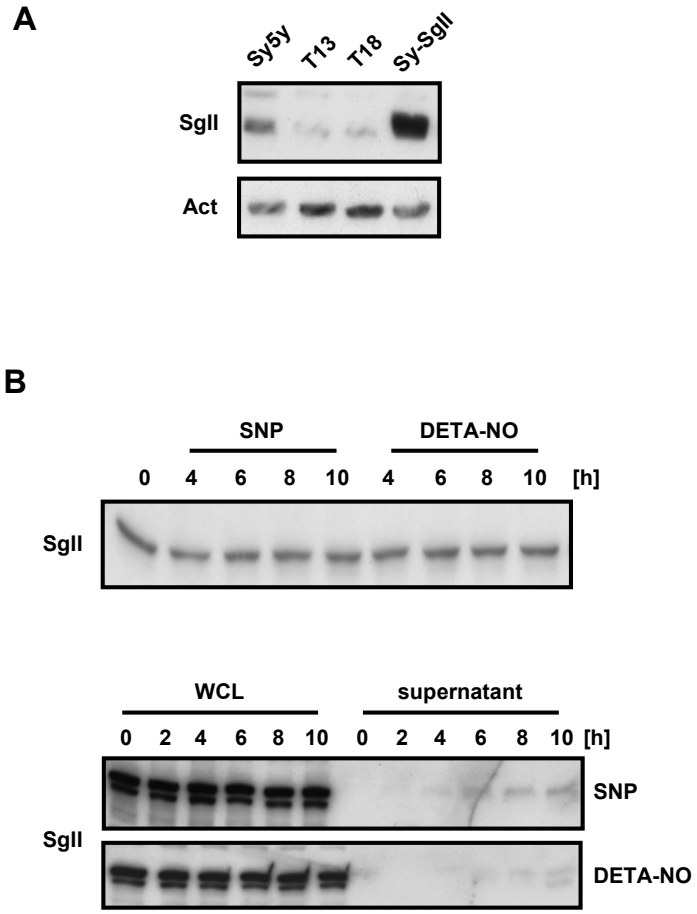

Fig 4.9 Basal and NO-inducible SgII protein levels in SH-Sy5y and TAM67 cells. (A) Total proteins were extracted from SH-Sy5y cells, two independent clones of TAM67 stable cells (T13 and T18) and one *sgII* over-expressing SH-Sy5y cell (Sy-SgII) were separated in 8%SDS-PAGE followed by Western blot analysis to detect SgII. (B) *Top panel*, SH-Sy5y cells were treated with 2 mM SNP or DETA-NO for the indicated times, and total proteins were extracted. *Bottom panel*, SH-Sy5y cells were treated with 2 mM SNP or DETA-NO for the indicated times. Total intracellular proteins as well as secreted proteins from the cell culture medium were extracted or collected, respectively. For both panels, the proteins were separated in 8%SDS-PAGE followed by Western blot analysis to detect SgII.

due to a time-dependent, NO-inducible release of SgII into the culture medium. (Fig 4.9B, *bottom panel*).

4.4 SgII plays important roles in neuroprotection in NO-induced apoptosis as well as in NGF-induced neuronal differentiation

Our laboratory has previously shown that c-Jun regulates NCAM140, and loss of NCAM140 in TAM67 cells partially sensitizes SH-Sy5y cells to apoptosis induced by NO and other oxidative stresses (Feng *et al*., 2002). To address whether SgII can also influence NO sensitivity, I stably reintroduced *sgII* cDNA (as a *sgII-myc* fusion) into TAM67 dominant-negative c-Jun cells, and obtained clones with varying degrees of SgII-Myc overexpression (Fig 4.10A, *left panel*) compared with the endogenous SgII expression levels (Fig 4.10A, *right panel*). Two independent SgII over-expressing clones both exhibited enhanced resistance to NO killing mediated by two different NO donors at various concentrations (Fig 4.10B), and caspase-3-like protease activity was correspondingly reduced in both clones (Fig 4.10D). Since caspase-3-like activity is required for NO-induced apoptosis, these data together suggest that SgII counteracts NO-mediated apoptosis of neuroblastoma cells.

As mentioned above, *sgV* was expressed in SH-Sy5y but not TAM67 cells (Fig 4.8B). However, in sharp contrast to *sgII*, stable expression of *sgV* in TAM67 cells failed to enhance resistance to NO donor-induced apoptosis (Fig 4.10C). This is evidence for a selective protective effect of SgII against NO toxicity compared with another member of the granin family. Our laboratory previously reported that c-Jun/AP-1 is required for NGF-induced neuronal differentiation, and TAM67 cells fail to undergo neuronal differentiation owing to lack of NCAM140 (Feng *et al*., 2002).

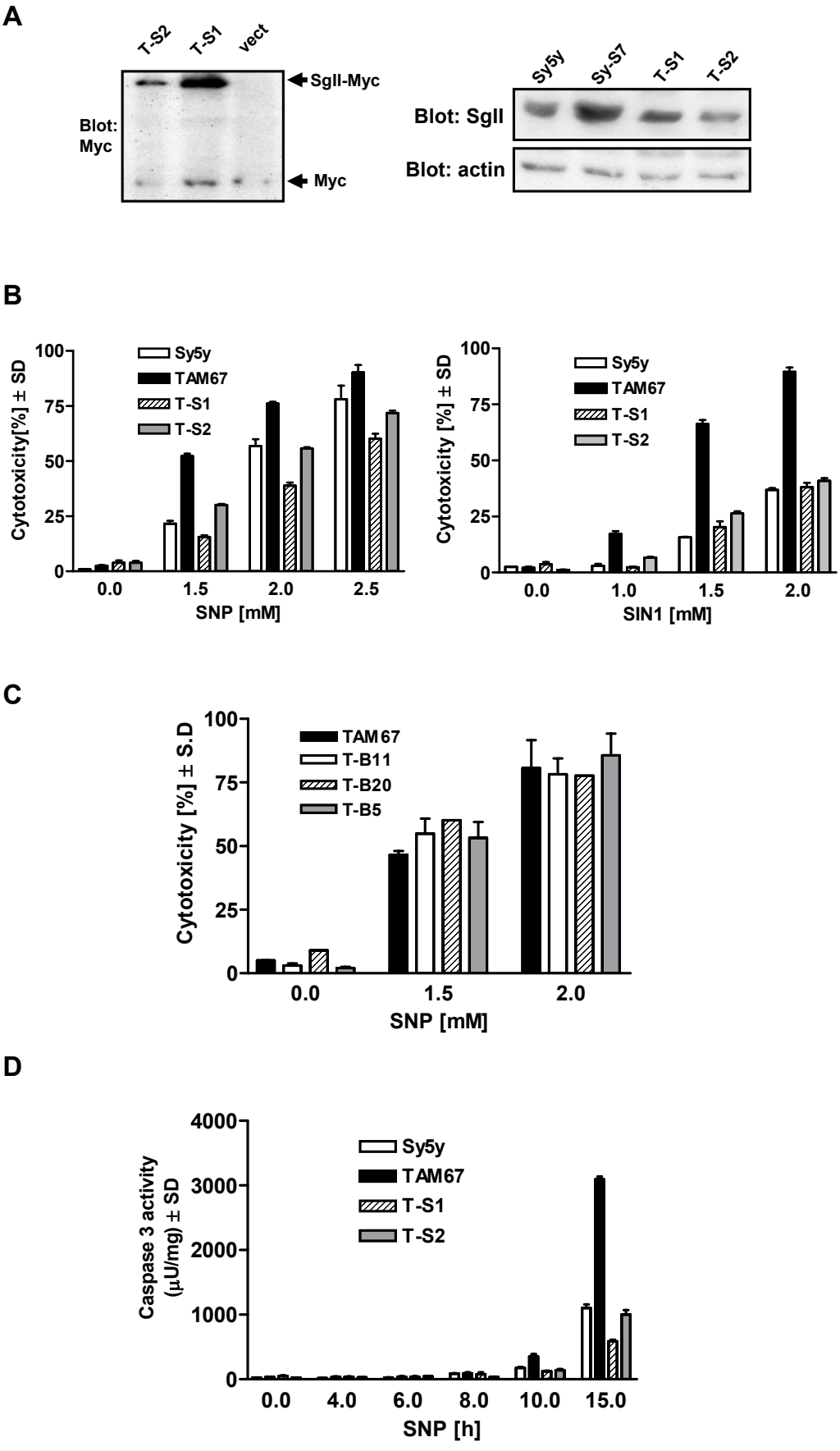
A
T-S2
T-S1
vect
SgII-Myc
Blot: Myc
Myc
Sy5y
Sy-S7
T-S1
T-S2
Blot: SgII
Blot: actin
B
Cytotoxicity[%] ± SD
Sy5y
TAM67
T-S1
T-S2
SNP [mM]
Cytotoxicity [%] ± SD
SIN1 [mM]
C
Cytotoxicity [%] ± S.D
TAM67
T-B11
T-B20
T-B5
SNP [mM]
D
Caspase 3 activity (µU/mg) ± SD
Sy5y
TAM67
T-S1
T-S2
SNP [h]

Fig 4.10. Increased NO-resistance of TAM-67 stable cells over-expressing sgII. (A) Over-expression of *sgII* in TAM-67 stable cells. *Right panel*, Western blot showing over-expression of *sgII* in TAM67 stable cells. The upper bands are SgII-c-Myc fusion proteins, and the lower bands represent endogenous c-Myc. *Right panel*, Western blot with SgII antibody detects the endogenous SgII in different cell lines. (B) SH-Sy5y cells, TAM-67 vector control cells and different *sgII* over-expressing TAM67 cell clones (T-S1, T-S2) were treated with different concentrations of SNP or SIN1 for 15 h, cells were harvested and the percentage of cell death was measured by LDH release assay. (C) TAM-67 cells and three different 7B2 overecpressing cells were treated with different concentrations of SNP for 15h, cells were harvested and percentage of cell death was measured by LDH release assay. (D) SH-Sy5y, T-S1 or T-S2, and TAM-67 vector control cells were treated with different concentrations of SNP for the indicated times, cells were harvested and caspase-3 activity was measured.

The next question was whether the *sgII*/TAM67 stable cells would regain competence for NGF-induced neuronal differentiation following NGF treatment for 8 days (aphidicolin was used together with NGF to enhance neuronal differentiation process by forcing cells exiting from cell cycle progression). Two independent clones of TAM67/*sgII* cells (T-S cells) as well as SH-Sy5y cells, but not TAM67 vector control cells, showed the classic neuronal differentiation morphology including neurite outgrowth, a network of interconnected neurons, and shrinkage of the cell bodies (Fig. 4.11A). It is established that Bcl-2 is strongly up-regulated during neuronal differentiation of some neurons and neuroblastoma cells (Feng and Porter, 1999; Raguenez *et al*., 1999); and in certain cell types Bcl-2 regulates neurite outgrowth and differentiation (Eom *et al*., 2004; Hilton *et al*., 1997). NGF induced a steady up-regulation of Bcl-2 in both *sgII*-over-expressing clones and SH-Sy5y cells, but not TAM67 stable cells that fail to differentiate (Fig 4.11B). Thus, SgII appears essential for NGF-induced neuronal differentiation by both morphological and biochemical criteria. In contrast, differentiation was not restored in TAM67 cells stably transformed

with *sgV*, indicating that SgV may not be required for neuronal differentiation (Fig 4.11C).

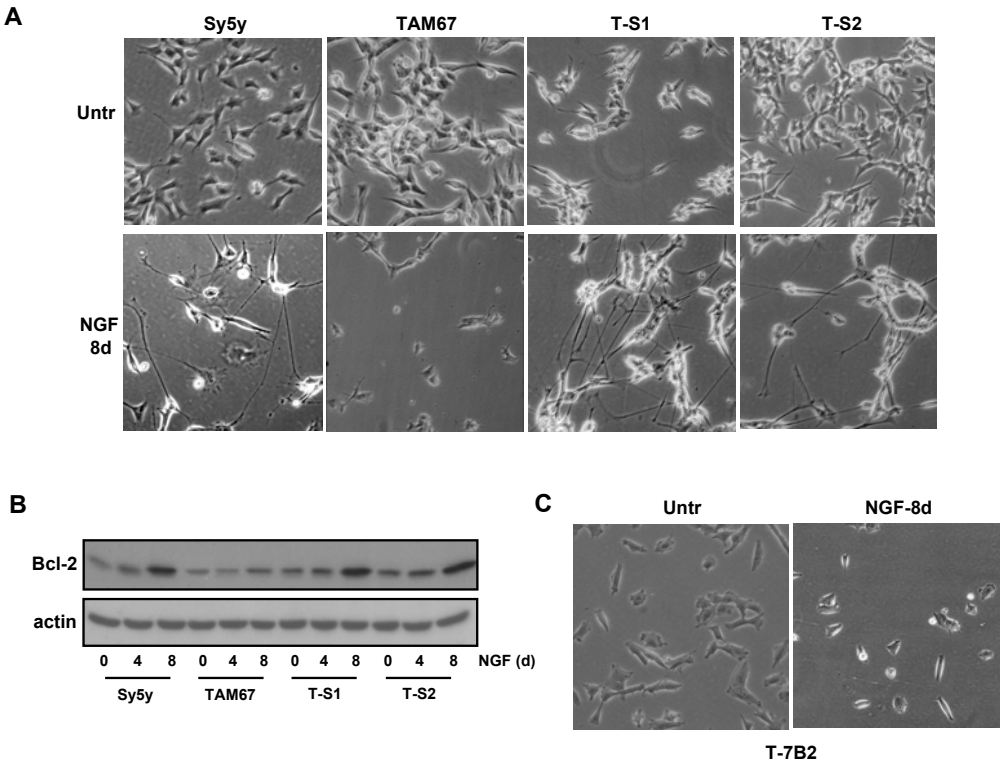

Fig 4.11 SgII over-expression restores neuronal differentiation in TAM67 cells. SH-Sy5y cells**,** TAM67 vector control cells and two independent clones of T-S stable cells (TAM67 cells over-expressing *sgII*) were induced into neuronal differentiation by NGF and alphidicolin for up to 8 days. (A) Morphological changes described in the text are shown before (untr) and after differentiation (NGF 8d). (B) Cells were harvested after the indicated number of days, and total proteins in the lysates were separated in 12% or 15% SDS-PAGE gels. Bcl-2 protein was revealed by Western blotting. Actin was the loading control. (C) Morphology of untreated and NGF-induced 7B2 overexpressing TAM-67 cells.

To answer the question what are the physiological functions of SgII in SH-Sy5y cells, I used the siRNA approach in which only *sgII* expression is disrupted while the other potentially protective proteins are still synthesized. Six different oligonucleotides were chosen to stably knocked down *sgII* (details described in the "materials and methods"), and independent SH-Sy5y stable cell lines were generated (Fig 4.12A). Independent clones derived from two of the oligonucleotides which demonstrated a 60-80% reduction in SgII protein (Fig. 4.12A) did not undergo NGF-mediated neuronal differentiation based on the morphological criteria (Fig 4.12B) and failure of Bcl-2 upregulation 8 days after NGF addition (Fig 4.12C), which is in contrast to the vector control cells. Interestingly, the undifferentiated *sgII* knockdown cells underwent massive cell death (Fig. 4.12B), as did NGF-treated SgII-deficient TAM67 cells (Fig. 4.11A). Moreover, reduction in SgII protein in two *sgII* knockdown clones sensitized SH-Sy5y cells to apoptosis induced by various concentrations of two different NO donors (Fig. 4.12D). These results provide additional evidence that SgII is important in countering NO toxicity as well as promoting NGF-induced neuronal differentiation in SH-Sy5y cells.

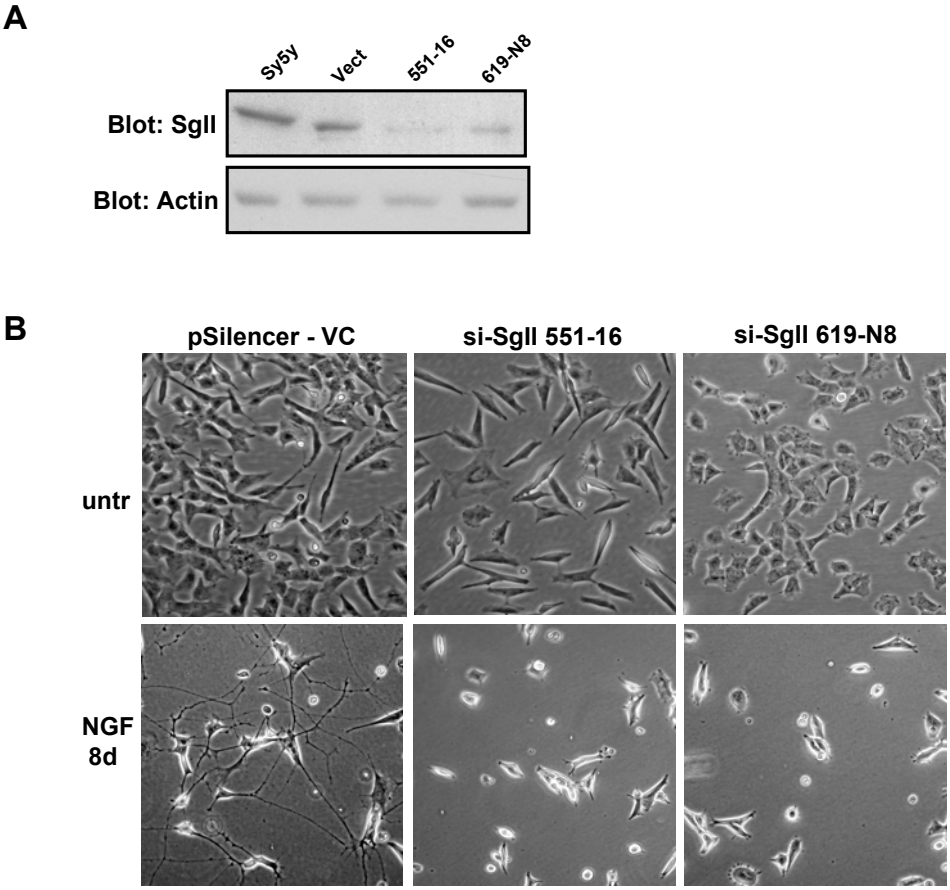
A
Sy5y
Vect
551-16
619-N8
Blot: SgII
Blot: Actin
B
pSilencer - VC
si-SgII 551-16
si-SgII 619-N8
untr
NGF
8d
C
Blot: Bcl-2
Blot: actin
0 4 8 0 4 8 0 4 8 NGF (d)
VC
551-16
619-N8
D
Cytotoxicity [%] ± SD
VC
551-16
619-N8
SNP [mM]
SIN1 [mM]

Fig 4.12 Knock-down of sgII expression inhibits neuronal differentiation and sensitizes SH-Sy5y cells to NO-induced apoptosis. (A) SH-Sy5y cells were stably transfected with p*Silencer–sgII* constructs, hygromycin-resistant clones were isolated (551-16 and 619-N8), and total proteins were subjected to electrophoresis in 8% or 12% SDS-PAGE gels. SgII and actin levels were detected by Western blotting. (B) Vector control (pSilencer-VC) cells and two independent clones of *sgII*-siRNA (551-16 and 619-N8) were subjected to NGF-induced neuronal differentiation for 8 days as described in "materials and methods". Morphological changes described in the text are shown before (untr) and after differentiation (NGF 8d). (C) Vector control (VC) and siRNA-expressing clones 551-16 and 619-N8 cells were treated with NGF for the indicated number of days, harvested and total proteins in the lysates were separated in 12% or 15% SDS-PAGE. Bcl-2 protein was revealed by Western blotting. Actin was the loading control. (D) Vector control (VC; open bars) cells and two different *sgII*-siRNA stable cell clones (551-16 and 619-N8) were treated with different concentrations of SNP (*left panel*) or SIN1 (*right panel*) for 15 h, and the percentage of cell death was determined by LDH release assay. Values are the mean ± S.D. determined from three experiments performed in triplicate.

4.5 Discussion

Proteins in the chromogranin family (collectively called granins) have a unique cellular localization in large dense core vesicles (LDCV's) in neurons and neuroendocrine cells. Early studies focused on their properties with relevance to secretion like calcium binding, aggregation at the TGN (trans-Golgi network), and sorting for entry into the regulated pathway of secretion (Fischer-Colbrie *et al*., 1995; Taupenot *et al*., 2003). Three classical proteins in this family, CgA, CgB and SgII, were shown to be stored in the LDCVs and released together with other peptide hormones and neurotransmitters upon stimulation. Besides their functions as helper proteins in protein sorting at the TGN, they themselves are precursors of certain biologically functional peptides, being subject to proteolytic cleavage within the LDCVs (Natori and Huttner, 1994; Taupenot *et al*., 2003). In addition, CgA may have a role in pro-apoptotic processes (Ciesielski-Treska *et al*., 1998; Ciesielski-Treska *et al*., 2001; Kingham and Pocock, 2000; Taylor *et al*., 2002). Thus, granins fulfill neuronal cell functions and have potential roles in regulating neuronal cell viability.

In this study, several novel findings were made. First, the expression and NO-mediated up-regulation of *sgII* depends to a large extent on c-Jun/AP-1, and requires a CRE element located just upstream of the *sgII* promoter (Scammell *et al*., 2000). *cgA, cgB* and *sgII* all bear similar conserved CRE elements in their promoter regions (Taupenot *et al*., 2003), yet only *sgII* is expressed and up-regulated by NO in a c-Jun/AP-1-dependent fashion. Because these conserved CRE elements dictate cell-type specific and inducible expression of granins, it is possible SH-Sy5y cells lack one or more additional factors needed for the CREs in the *cgA* and *cgB* promoters to respond to NO. Other novel findings in my study are that SgII (but not the family member SgV) mediates NGF-stimulated neuronal differentiation and protection from NO-induced apoptosis in a c-Jun/AP-1-dependent manner. These functions of SgII were rigorously demonstrated with two opposite but complementary approaches. In one, SgII synthesis was selectively restored in TAM67 cells that express very low levels of *sgII*. In another approach, *sgII* was specifically knocked down with siRNA in cells with normal c-Jun/AP-1 function. Incomplete knockdown of *sgII* was sufficient to compromise neuronal differentiation and sensitize the cells to apoptosis, suggesting that even a modest reduction in the expression of SgII can affect cell differentiation and viability. Unlike SH-Sy5y cells, SgII-deficient TAM67 cells and *sgII* knockdown SH-Sy5y cells failed to differentiate and underwent massive cell death after NGF treatment, implying that SgII might also be neuroprotective during neuronal differentiation. From our previous work, NCAM140 is also essential for NGF-dependent neuronal differentiation and protection from NO-induced apoptosis of neuroblastoma cells (Feng *et al*., 2002). c-Jun/AP-1-inhibited TAM67 cells lack NCAM140 protein, which was restored by *sgII* expression (unpublished observations), implying that *sgII* over-expression can up-regulate NCAM140 in a common survival/ differentiation pathway.

Overall my results indicate that SgII can mediate neuronal differentiation and protection from apoptosis even when other c-Jun/AP-1-regulated genes are repressed.

How might SgII fulfill a dual function in neuroprotection and neuronal differentiation? SgII is the precursor of the peptide secretoneurin (SN), which is secreted and is consequently involved in many cellular processes including neurotransmitter release (Agneter *et al*., 1995; Saria *et al*., 1993), proliferation (Kahler *et al*., 1997a; Kahler *et al*., 1997b), and survival of cerebellar granule cells (Fujita *et al*., 1999). Although a specific SN receptor has not been identified, there is evidence suggesting that SN might act *via* a classical G-protein-coupled receptor (Gasser *et al*., 2003b; Kahler *et al*., 2002). Ligation of G-protein-coupled receptors is known to activate the MAP kinase cascade (Chakraborty, 2001) resulting in cell differentiation, survival or death (Schaeffer and Weber, 1999). *sgII* transcriptional activation was reported in neuronal cells challenged with different stresses (Chiang *et al*., 2001; Li *et al*., 2002). In one study, *sgII* upregulation was dependent on phosphatidyl-inositol-3-kinase (PI3K) in response to oxidative stress (Li *et al*., 2002). Since PI3K is a well-established survival signal transducer in neurons, it is conceivable that SgII is an important component of the PI3K-dependent survival pathway. Thus, SgII might mediate protection from NO directly or indirectly *via* secreted SN in an autocrine and paracrine fashion. Detection of secreted SgII is consistent with the former possibility. The presence of SgII in the growth medium might also explain why NO stimulated an increase in *sgII* mRNA but not intracellular SgII protein.

Several reports proposed SgII as a marker of neuronal differentiation, based on observations that SgII is upregulated at the transcriptional and translational levels in several systems (Laslop and Tschernitz, 1992; Weiler *et al*., 1990b). In differentiated neuroblastoma cells, SgII was present in the Golgi and at the periphery of neurites and

in growth cones, but was virtually undetectable in undifferentiated cells (Giudici *et al*., 1992). However, neither CgA nor CgB was found co-localized with SgII in the same organelles in differentiated cells. SgII always shows higher expression levels in different cells of the neuronal type or neural precursor cells (Hagn *et al*., 1986; Laslop and Tschernitz, 1992; Weiler *et al*., 1990a). Moreover, SN may contribute to neurite outgrowth of cultured neuronal cells (Gasser *et al*., 2003). Thus, NGF on the one hand might trigger differentiation-inducing signal transduction pathways leading to activation of transcription factors and their target genes including *sgII*. On the other hand, NGF could also promote the release of various components from LDCVs such as SN, resulting in the secondary activation of signal transduction pathways required for neuronal differentiation.

CHAPTER 5 How opposite functions of AP-1 factors are achieved in a single cell line

In chapter 3, I showed that phospho-c-Jun played a pro-apoptotic role in NO-induced apoptosis in SH-Sy5y cells based on the discovery that JunAA/S63A stable cells are more resistant to NO. In chapter 4, an anti-apoptotic function of c-Jun/AP-1 in response to the same stimuli is revealed based on the observation that TAM67 stable cells are more sensitive to NO. It is important to explain the opposing effects of the dominant-negative c-Jun (TAM-67) and S63A/ JunAA in an attempt to understand the role(s) of c-Jun/AP-1 in NO-induced apoptosis.

5.1 Structural differences between TAM67 and JunAA/S63A determines their functional discrepancy

Firstly, it is necessary to examine the structural difference between the two dominant-negative mutants of c-Jun (TAM67 and JunAA/S63A). In the TAM67 mutant, most of the transactivation domain of c-Jun is deleted, while in the JunAA/S63A mutant, the transactivation domain of c-Jun is intact except for one or two point mutations (both Ser-63 and Ser-73 are mutated to Ala or only Ser63 is mutated to Ala) as shown in Fig 5.1. From earlier studies, phosphorylation of Ser-63 and Ser-73 is essential for full activation of c-Jun with various stimuli (Behrens *et al*., 1999; Smeal *et al*., 1991). However, phosphorylation of these two residues is dispensable for basal c-Jun activity. Support for a phosphorylation-independent function of c-Jun comes from several other directions. For example, mutant mice in which the c-Jun locus is replaced by JunAA are healthy and fertile although they are obviously smaller in size than their wild type counterparts (Behrens *et al*., 1999), in

contrast to the embryonic lethality of $Jun^{-/-}$ mice (Johnson *et al.*, 1993), suggesting JunAA retains basal c-Jun activity despite the fact that its full activity is compromised. In addition, JunAA itself can regulate transcription by acting as a suppressor and antagonist of other transcription factors (Herdegen and Waetzig, 2001).

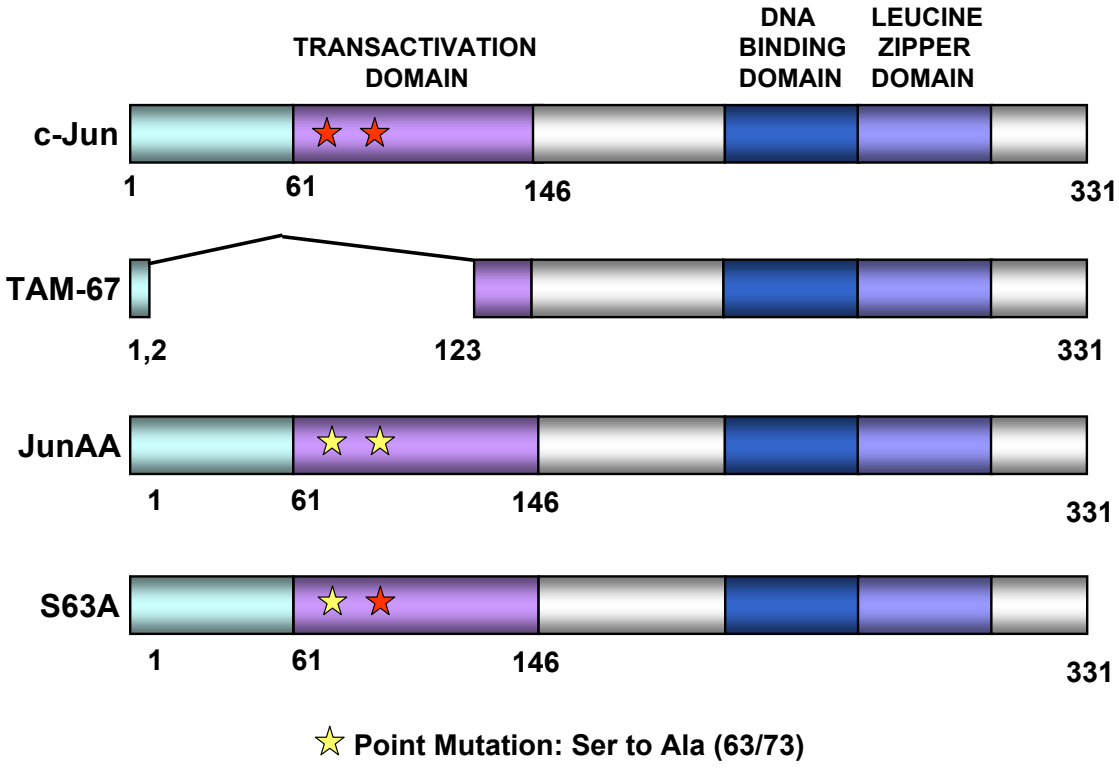

Fig 5.1 Comparison of structures of wild type c-Jun, TAM67 and JunAA/S63A

Thus, the structural difference between TAM67 and JunAA might explain the different phenotypes of the respective dominant-negative cells. Over-expressed TAM67 could potentially inhibit general AP-1 mediated transcription (Brown *et al.*, 1994; Thompson *et al.*, 2002) since c-Jun is an essential component of AP-1 dimers.

On the contrary, JunAA expression would only quench phospho-c-Jun mediated events which result from external stimuli, leaving the basal c-Jun-dependent events unaffected. This is proven by reporter analysis comparing the basal AP-1 activity in wild type SH-Sy5y cells, TAM67 stable cells and S63A stable cells. As shown in Fig 5.2, TAM67 over-expression resulted in dramatic inhibition of AP-1-mediated transcription in SH-Sy5y cells, while S63A expression slightly enhanced AP-1-mediated transcription, probably due to a slight excess of S63A in the cells (Fig 3.4A). However, over-expression of JunAA/S63A inhibited NO-induced c-Jun activity which is dependent on the phosphorylation of c-Jun on Ser-63 (Fig 3.6A), suggesting the non-phosphorylated mutant of c-Jun (JunAA/S63A) is able to counteract the deleterious effects arising from c-Jun phosphorylation.

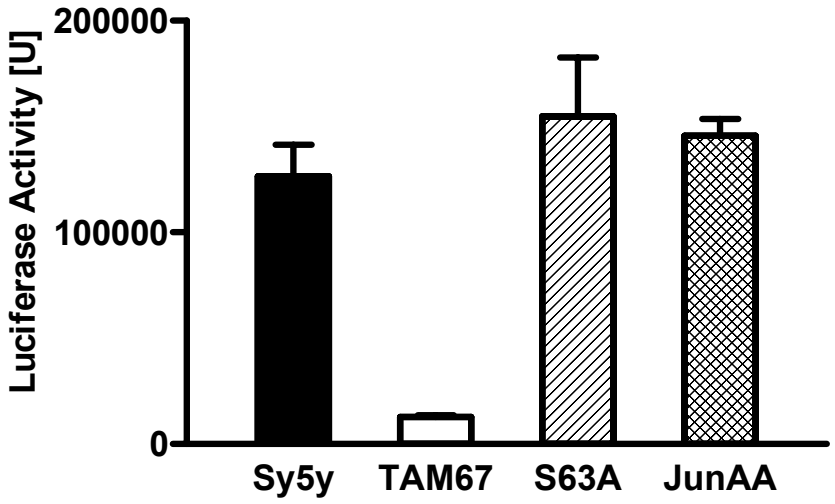

Fig 5.2 Comparison of AP-1 activity in wild type SH-Sy5y cells, TAM67 stable cells and JunAA/S63A stable cells. The TRE-reporter plasmid was cotransfected with the RPL-TK plasmid into SH-Sy5y, TAM67, S63A and JunAA stable cells. 40 h later, the cells were harvested. Firefly luciferase activity was measured, and Renilla luciferase activity was measured as an internal control.

It is concluded that TAM67 interferes with most c-Jun/AP-1 dependent transcription events irrespective of the c-Jun phosphroylation, while JunAA/S63A specifically inhibits phospho-c-Jun mediated events.

5.2 Molecular mechanisms responsible for the apparent differences between TAM67 and JunAA/S63A stable cells

Since basal AP-1 activity in TAM67 and JunAA/S63A stable cells is different, it is likely that this accounts for the huge difference in their sensitivity to NO.

It has been previously shown by my laboratory that the synthesis of NCAM140, a neuroprotective protein, is AP-1 dependent in SH-Sy5y cells (Feng *et al*., 2002). NCAM (neural cell adhesion molecule) belongs to the immunoglobulin superfamily, and three major isoforms may be generated from a single gene by alternative splicing (Doherty and Walsh, 1992; Tacke and Goridis, 1991). Using primers specific for exon 18 of NCAM, a unique exon present in *NCAM180* transcripts but not in *NCAM140* transcripts (Fig 5.3A), it was found that *NCAM180* is only expressed in TAM67 cells (Fig 5.3C). Consistently, different patterns of NCAM140 and NCAM180 synthesis in vector control and TAM67 stable cells were also observed (Fig 5.3B). NCAM140 synthesis is not dependent on c-Jun phosphorylation since a basal level of NCAM140 is maintained without NO treatment, which decreases with NO stimulation (Fig 5.4A). Furthermore, NCAM140 counteracts NO toxicity. When a cDNA encoding NCAM140 was stably transfected into TAM67 cells (NCAM140 negative), cells showed a marked resistance to NO-induced apoptosis compared with TAM67 cells (Feng *et al*., 2002)(Fig 5.4B).

However, in JunAA and S63A stable cells, in which basal AP-1 activity is still normal, both the transcription of *NCAM140* and the synthesis of NCAM140 are

unaffected (Fig 5.5 A, B), suggesting the NCAM140-mediated survival pathway is intact in these cells.

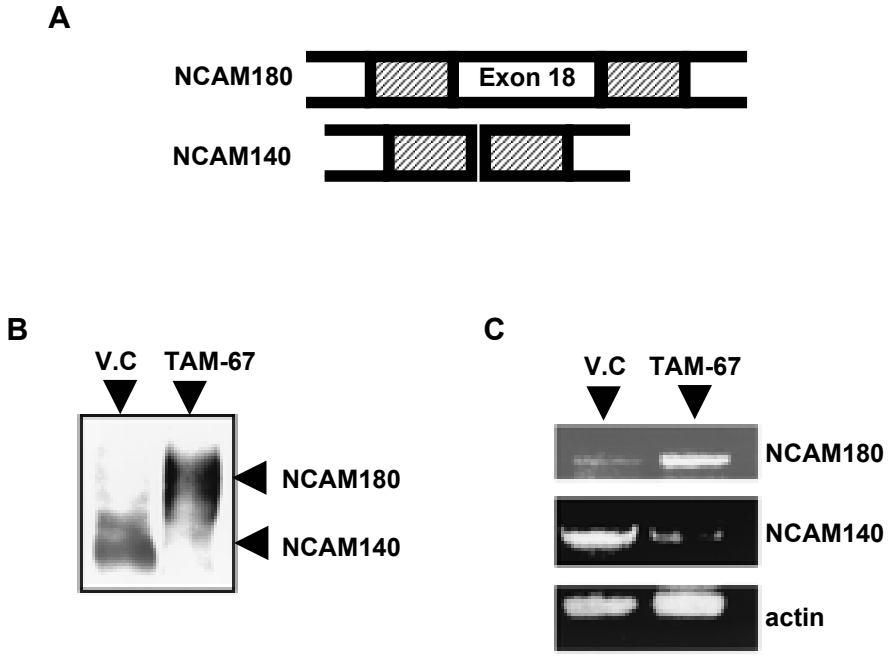

Fig 5.3 NCAM140 synthesis is AP-1 dependent in SH-Sy5y cells. (A) Comparison of mRNA structure of *NCAM140* and *NCAM180*. (B) Total proteins from vector control and TAM67 stable cells were extracted and subjected to 6% SDS-PAGE followed by western blot analysis using an antibody against NCAM. (C) Total RNA was extracted from vector control and TAM67 stable cells. RT-PCR detected *NCAM180* synthesis (using a primer specific for exon 18) and *NCAM140* synthesis. Actin was also visualized to confirm equal input of RNA (Feng *et al*., 2002).

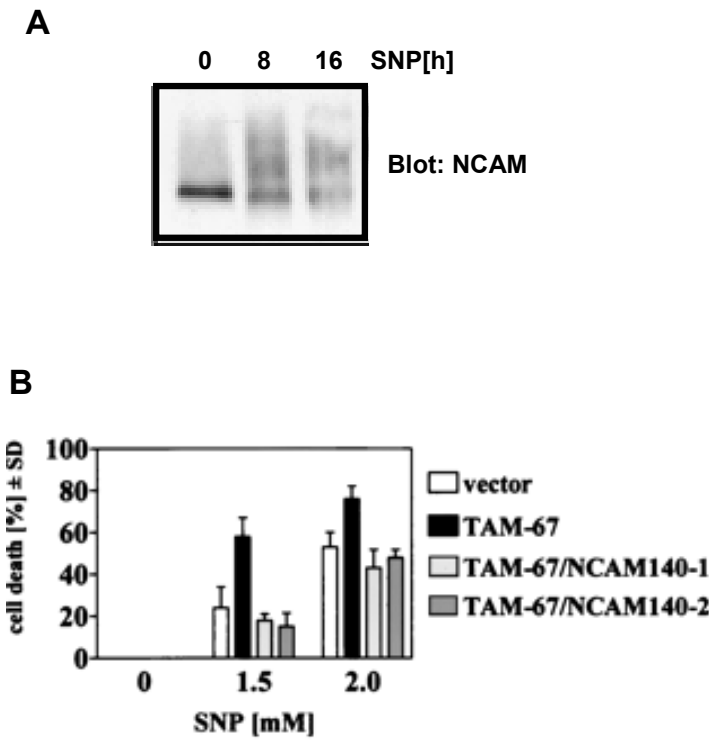

Fig 5.4 NCAM140 protects SH-Sy5y cells from NO-induced apoptosis. (A) NCAM140 synthesis is not dependent on NO-induced phosphorylation of c-Jun. SH-Sy5y cells were treated with 2 mM SNP for the indicated times and total proteins were extracted and subjected to 6% SDS-PAGE followed by westwen blot analysis using an NCAM antibody. (B) TAM67 stable cells, TAM67 cells overexpressing NCAM140 and their vector control cells were treated with 2mM SNP for 15 h, and the percentage of cell death was measured by crystal violet staining (Feng *et al*., 2002).

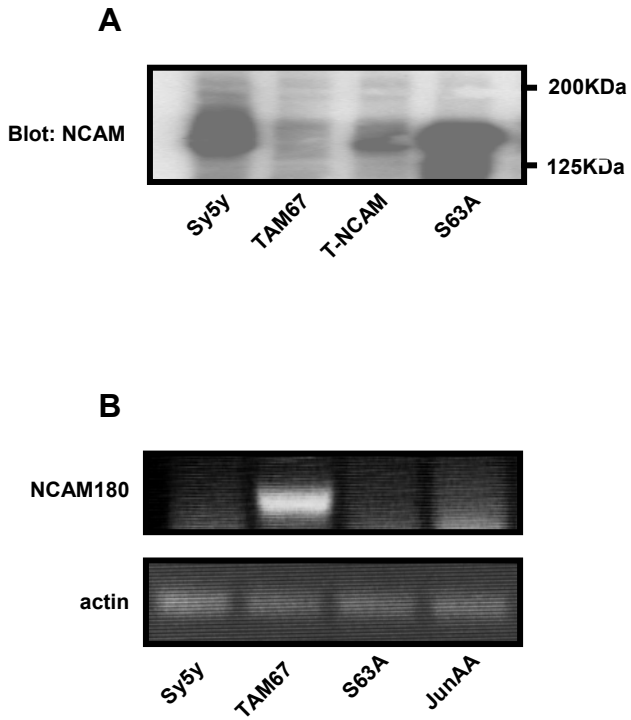

Fig 5.5 NCAM140 expression is intact in JunAA/S63A stable cells. (A) Total proteins from SH-Sy5y, TAM67, S63A as well as NCAM140 over-expressing TAM67 cells (T-NCAM) were prepared and subjected 6% SDS-PAGE followed by western blot analysis using an antibody against NCAM. (B) Total RNA was extracted from SH-Sy5y, TAM67, S63A and JunAA stable cells. RT-PCR was done to detect *NCAM180* transcripts by using a primer specific for exon 18 of the *NCAM* gene (a unique exon present in *NCAM180* transcripts).

In chapter 4, I discussed the neuroprotective gene *sgII* whose expression is AP-1 dependent, because basal and inducible *sgII* transcription and basal SgII translation were severely compromised in TAM67 stable cells. Since JunAA/S63A over-expression did not affect the basal activity of AP-1 in SH-Sy5y cells, I also checked the expression of *sgII* in these cells. In sharp contrast to TAM67 cells, *sgII* transcription and translation in JunAA/S63A cells was similar to wild type SH-Sy5y

cell levels (Fig 5.6A, B), indicating a SgII-mediated survival pathway is also intact in these cells.

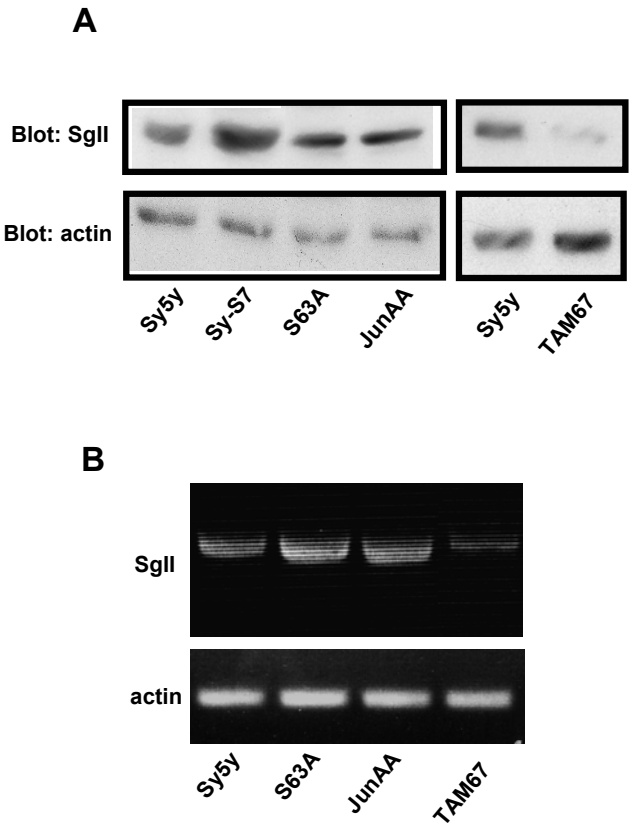

Fig 5.6 SgII expression is intact in JunAA/S63A stable cells. (A) Total proteins from SH-Sy5y, *sgII*-overexpressing SH-Sy5y cells (Sy-S7), S63A, JunAA and TAM67 stable cells were prepared and subjected 8% SDS-PAGE followed by western blot analysis using an antibody against SgII. (B) Total RNA was extracted from SH-Sy5y, S63A, JunAA and TAM67 stable cells. RT-PCR was done using primers specific for *sgII*.

A speculative model is presented in Fig. 5.7 that can help to understand NO-activated transcription in SH-Sy5y cells and how the two different dominant-negative

forms of c-Jun might affect transcription. The TAM-67 dominant-negative protein is able to efficiently inhibit general AP-1-mediated transcription including the expression of certain neuroprotective genes (*NCAM140* and *sgII*); therefore, the cells become more sensitive to NO. The synthesis of NCAM140 and SgII does not presumably require Ser-63 phosphorylation of c-Jun, because it occurs in the absence of NO stimulation, which is essential to activate the JNK-phospho-c-Jun pathway. In contrast to TAM-67 cells, NCAM140 and SgII protein are still synthesized at normal levels in NO-resistant S63A and JunAA cells. This indicates the constitutive c-Jun/AP-1-dependent NCAM140 / SgII survival pathway(s) are intact in these cells. In other words, the S63A and JunAA (and related constructs) block the pro-apoptotic JNK-c-Jun pathway without affecting the synthesis of neuroprotective NCAM-140 and SgII, so the cells are resistant to apoptosis compared to SH-Sy5y cells and TAM-67 cells. *DN-jnk* could achieve similar effects through inhibition of JNK and thus block c-Jun phosphorylation.

Consistent with the studies shown in this thesis, it was reported that c-Jun can protect undifferentiated rat PC12 neuronal cells from apoptosis independently of c-Jun phosphorylation, but in the fully differentiated cells JNK signaling can induce apoptosis and c-Jun mediates this response (Leppa *et al*., 2001). However, NO was not one of the apoptosis paradigms used. Based on the current study, it is evident that c-Jun/AP-1 can fulfill opposite functions in a single undifferentiated neuroblastoma cell, which I speculate occurs in the following context. A constitutive or basal activity of c-Jun/AP-1 factor(s) (independent of c-Jun phosphorylation on Ser-63) is able to counteract relatively low levels of NO - in part through the constant expression of neuroprotective NCAM140 and SgII. In contrast, a toxic concentration of NO will lead

to c-Jun phosphorylation on Ser-63 by JNK that triggers apoptosis *via* yet discovered c-Jun targets.

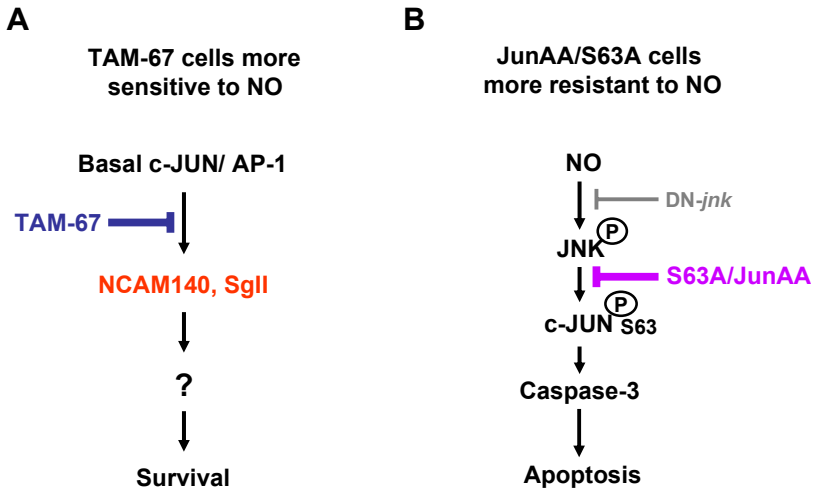

Fig 5.7 Speculative model of how different dominant-negative forms of c-Jun (TAM-67 and S63A/ JunAA) have opposite effects on the sensitivity of SH-Sy5y cells to NO. (***A***) TAM-67 over-expression in SH-Sy5y cells leads to a general suppression of c-Jun/AP-1-dependent transcription, including protective genes encoding NCAM140 and SgII, regardless of phosphorylation of c-Jun on Ser-63 and Ser-73. Therefore, TAM-67 cells are sensitized to apoptosis. (***B***) S63A and JunAA only block pro-apoptotic phospho-c-Jun dependent events such as caspase-3 activation, whereas the phospho c-Jun-independent neuroprotective NCAM140 is still synthesized in the S63A stable cells. Therefore, S63A and JunAA cells are more resistant to apoptosis than SH-Sy5y cells. DN-*jnk* (*-1 or -2*) inhibits the activity of JNK, leading to the failure to phosphorylate c-Jun, which therefore has the same effect as S63A and JunAA.

CHAPTER 6 Implications and Future Prospects

Nitric oxide (NO) is an important molecule mediating various physiological as well as pathophysiological processes. NO has received more attention in recent years, since it is functionally involved in the pathology of acute or chronic neurological disorders (Lipton, 1999). Furthermore, due to the complex chemical reactions of NO, it is potentially implicated in the process of aging. It is relevant to understand the mechanisms whereby NO exerts its notorious effects on cells and what defensive responses the cells can activate to counteract NO toxicity. AP-1 was among the first identified mammalian transcription factors, but the physiological functions are still far from elucidated. The complexity of AP-1 functions is believed to arise from the different compositions of AP-1 dimers and different sets of target genes activated by AP-1 in cell-, development stage- or stimulus-specific manners. Identification of these targets genes will be helpful in understanding the diverse AP-1 functions in different situations (Karin *et al*., 1997). In the current study, I investigated two distinct but interconnected events elicited by AP-1 in response to NO: a pro-apoptotic event activated by AP-1 due to c-Jun phosphorylation and an anti-apoptotic pathway activated by AP-1 due to expression of two neuroprotective genes.

It is established that phosphorylation of Ser-63 and Ser-73 on c-Jun usually enhances its transcriptional activity. However, it was not previously addressed whether single phosphorylation of one or the other of these two residues might occur under some circumstances and if so, what might be the the physiological relevance?

In the current study, I found Ser-63 of c-Jun to be heavily phosphorylated upon NO stimulation in two neuroblastoma cells (SH-Sy5y and SHEP cells) while the Ser-73 phosphorylation is marginally detectable. Further studies proved that this

phosphrylation is mediated by JNK and not p38. What is the mechanism underlying the single phosphorylation of c-Jun? MAPKs are highly specific in their choice of substrates. In the case of JNK and c-Jun, efficient phosphorylation of c-Jun by JNKs requires a docking site located between amino acids 30 and 60 of c-Jun, a proline residue following the serine residue to be phosphorylated as well as specific residues flanking the phosphoacceptor site (Karin, 1995). The first possibility is that c-Jun is defective in the structure mentioned above in the two cell lines I used. Strong phosphorylation on both Ser-63 and Ser-73 of c-Jun upon UV irradiation argues that c-Jun is, however, intact in this region. Another possibility is that NO activates a specific JNK isoform which has higher affinity for Ser-63 than Ser-73. JNK2 has higher affinity to c-Jun than JNK1 because of a small region near its catalytic pocket (Kallunki *et al*., 1994). It is reasonable to speculate that unique or particular structural features of different JNK isoforms might determine their preference for their substrates. Further experiments can be done to study which JNK isoform(s) have been activated by NO and further analyze their contributions to c-Jun phosphorylation. Cells from mice deficient for particular JNK isoforms might also be useful to address this question. A third possibility is that NO might activate specific phosphatases which specifically recognize and rapidly dephosphorylate phosphor-Ser-73. Known phosphatase inhibitors might be useful in testing this hypothesis. A fourth possibility is that putative NO-mediated chemical modification (*e.g.* nitrosation) of c-Jun could preferentially block Ser-73 phosphorylation.

I have shown predominant Ser-63 phosphorylation of c-Jun in response to NO is functionally important; it enhances c-Jun as well as AP-1 transcriptional activity in SH-Sy5y cells; it also results in apoptosis in both SH-Sy5y and SHEP cells. What is the mechanism of phospho-c-Jun in mediating apoptosis? Besides contributing to

caspase-3 activation (most probably indirectly), phospho-c-Jun (S63) might induce a different spectrum of target genes compared with the un-phosphorylated c-Jun or dual-phosphorylated c-Jun, resulting in a shift to an overwhelming expression of death genes. A recent publication reports the sole phosphorylation of c-Jun on Ser-73 in cultured sympathetic neurons deprived of glial cell line–derived neurotrophic factor (GDNF) (Yu *et al*., 2003). However, the biological significance of c-Jun phosphorylation on Ser-73 in GDNF withdrawal-induced cell death is not clear. Importantly, another publication reports that absence of c-Jun phosphorylation on Ser-63 in response to an oxidative stress-inducer (3-NPA) might play a role in slowing the aging process in Snell Dwarf mice (Madsen *et al*., 2004). It is intriguing but remains to be determined whether slower aging is connected to lower levels of oxidative stress and apoptosis resulting from lack of Ser-63 phosphorylation. These observations provide additional evidence that under certain circumstances, phosphorylation of c-Jun on either Ser-63 or Ser-73 can occur and may have functional relevance. Comparing the gene expression profiles when c-Jun is un-phosphorylated, dual-phosphorylated and S63/S73-phosphorylated will be helpful in understanding the molecular mechanisms underlying these different events.

The role of AP-1 in apoptosis is complex; its activation can be proapoptotic, antiapoptotic or irrelevant to apoptosis. The precise function of AP-1 and actual cell fate in response to a specific stimulus is dependent on the cellular context in which AP-1 is activated, including co-activation of other signaling molecules which might directly or indirectly affect AP-1 activity, activation of the dimerization components of AP-1 which will determine the spectrum of AP-1 target genes (Chinenov and Kerppola, 2001). In the current studies, I notably found that c-Jun/AP-1 can fulfill both anti- and pro-apoptotic functions in a single cell line in response to a single stimulus. Consistent

with these studies, it was reported that c-Jun can protect undifferentiated rat PC12 neuronal cells from apoptosis independently of c-Jun phosphorylation, but in the fully differentiated cells JNK signaling can induce apoptosis and phosphorylated c-Jun mediates this response (Leppa *et al*., 2001). The former event does not seem to depend on c-Jun transcriptional activity while the latter one does. Based on my studies presented here, both pro- and anti-proapoptotic activity of c-Jun/AP-1 are apparently mediated by its transcription activity, which might occur in the following context: a constitutive or basal activity of c-Jun/AP-1 factor(s) (independent of c-Jun phosphorylation on Ser-63) is able to counteract relatively low levels of NO - in part through the constant expression of neuroprotective NCAM140 and SgII. In contrast, a toxic concentration of NO will lead to c-Jun phosphorylation on Ser-63 by JNK that triggers apoptosis *via* yet discovered c-Jun targets. It is also possible that other transcription factors activated by NO can affect AP-1 activity and cell viability. My colleagues have demonstrated that activation of the transcription factor Nrf2 counteracts NO toxicity (Dhakshinamoorthy and Porter, 2004). According to my preliminary unpublished results, Nrf2 is able to suppress JNK-c-Jun signaling. Thus, in Nrf2 over-expressing SH-Sy5y cells, death is prevented partially due to the postponement of the NO-induced phosphorylation of c-Jun. Conversely, in SH-Sy5y cells over-expressing DN-Nrf2 or in Nrf2 knockdown cells, the increase in NO-induced c-Jun transcriptional activity starts at an earlier time point, suggesting an earlier activation of JNK-c-Jun signaling, and consequently increased apoptosis. It will be important to investigate how Nrf2 delays c-Jun phosphorylation.

It is also important to note that the non-phosphorylated mutant of c-Jun (JunAA/S63A) efficiently prevents NO-induced cell death while leaving the basal c-Jun/AP-1 dependent transcription (*e.g.* NCAM140, SgII) unaffected. Since

phosphorylation of c-Jun mostly occurs when cells are challenged with stress which might lead to cell death, inhibition of c-Jun Ser-63/Ser-73 phosphorylation could be a potential therapeutic approach when prevention of cell death is required, such as in acute or chronic neurological disorders.

Identification of pathway-specific AP-1 target gene(s) is essential in understanding the diverse functions of AP-1 achieved under different circumstances. Among the chromogranin family genes, *sgII* is uniquely responsive to NO in SH-Sy5y cells. Furthermore, basal expression of *sgII* also relies heavily on AP-1 factors through the conserved CRE motif within its promoter region. These observations help to explain the protective role of AP-1 in countering NO toxicity in SH-Sy5y cells. Although it is unclear how the dual functions of SgII (neuroprotection and neuronal differentiation) are achieved, there are some clues that the biologically functional peptides derived from SgII cleavage might account for it (Taupenot *et al*., 2003). SN (secretoneurin) is the best characterized peptide derived from SgII, involved in various biological processes including stimulation of proliferation and promotion of neurite outgrowth in certain cell types (Taupenot *et al*., 2003; Wiedermann, 2000). A simple way to test whether SN is responsible for SgII-mediated survival and neuronal differentiation is to block SN function by applying either anti-SN serum or pertussis toxin (PTX) (Gasser *et al*., 2003) on SH-Sy5y cells. If the cells were sensitized to NO-induced cell death or unable to undergo NGF-induced neuronal differentiation, SgII might function through SN. Involvement of other SgII-derived peptides in fulfilling SgII functions cannot be excluded. SH-Sy5y cells either over-expressing non-cleavable SgII or bearing non-functional PCs (endoproteinases responsible for SgII cleavage) (Fischer-Colbrie *et al*., 1995) will be helpful to answer whether SgII cleavage is important for its functions.

Another intriguing finding is the re-synthesis of NCAM140 in TAM67 cells over-expressing SgII (my unpublished observations). This observation places the NCAM140 and SgII in a possibly common regulatory pathway. NCAM140 is a trans-membrane protein (Cunningham *et al*., 1987), usually targeted to the plasma membrane through the constitutive secretory pathway after synthesis; SgII is a classical protein located in the regulated secretory pathway although there is evidence SgII may localize in the constitutive secretory pathway as well in certain cell types (Fischer-Colbrie *et al*., 1995). Three NCAM isoforms (120, 140 and 180 KDa respectively) arise from post-transcriptional splicing (Cunningham *et al*., 1987; Tacke and Goridis, 1991). It is not clear how SgII could affect the splicing process, an event occurring in the nucleus. However, NCAM140 synthesis is intact in SgII knockdown cells (my unpublished observations), suggesting SgII is not essential for *NCAM* splicing. Conversely, NCAM140 synthesis is completely compromised in TAM67 stable cells (Feng *et al*., 2002), indicating an essential role of AP-1 in *NCAM* splicing. Based on the experimental results, SgII seems required but not sufficient for NCAM140 synthesis in SH-Sy5y cells, which might happen in the following contexts: redundant pathways for NCAM140 synthesis exists in SH-Sy5y cells: one is dependent on AP-1 activity (probably through regulating the production or activity of splicing factors) and it is a predominant pathway (Feng *et al*., 2002). So long as normal AP-1 activity is present in the cells, NCAM140 is synthesized; another pathway is dependent on SgII and it is a minor pathway, functioning only when AP-1 activity is severely affected. Many more experiments need to be done to test this hypothesis.

Reference List

Adams,J.M. and Cory,S. (1998). *The Bcl-2 protein family: arbiters of cell survival*. Science *281*, 1322-1326.

Agneter,E., Sitte,H.H., Stockl-Hiesleitner,S., Fischer-Colbrie,R., Winkler,H., and Singer,E.A. (1995). *Sustained dopamine release induced by secretoneurin in the striatum of the rat: a microdialysis study*. J Neurochem *65*, 622-625.

Ameyar,M., Wisniewska,M., and Weitzman,J.B. (2003). *A role for AP-1 in apoptosis: the case for and against*. Biochimie *85*, 747-752.

Andrecht,S., Kolbus,A., Hartenstein,B., Angel,P., and Schorpp-Kistner,M. (2002). *Cell cycle promoting activity of JunB through cyclin A activation*. J Biol Chem *277*, 35961-35968.

Aronheim,A., Zandi,E., Hennemann,H., Elledge,S.J., and Karin,M. (1997). *Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions*. Mol Cell Biol *17*, 3094-3102.

Bakiri,L., Lallemand,D., Bossy-Wetzel,E., and Yaniv,M. (2000). *Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression*. EMBO J *19*, 2056-2068.

Barton,K., Muthusamy,N., Chanyangam,M., Fischer,C., Clendenin,C., and Leiden,J.M. (1996). *Defective thymocyte proliferation and IL-2 production in transgenic mice expressing a dominant-negative form of CREB*. Nature *379*, 81-85.

Behrens,A., Sibilia,M., and Wagner,E.F. (1999). *Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation*. Nat. Genet. *21*, 326-329.

Blendy,J.A., Kaestner,K.H., Weinbauer,G.F., Nieschlag,E., and Schutz,G. (1996). *Severe impairment of spermatogenesis in mice lacking the CREM gene*. Nature *380*, 162-165.

Bogdan,C. (2001). *Nitric oxide and the regulation of gene expression*. Trends Cell Biol. *11*, 66-75.

Bonny,C., Oberson,A., Negri,S., Sauser,C., and Schorderet,D.F. (2001). *Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death*. Diabetes *50*, 77-82.

Boyd,C.S. and Cadenas,E. (2002). *Nitric oxide and cell signaling pathways in mitochondrial-dependent apoptosis*. Biol Chem *383*, 411-423.

Boyle,W.J., Smeal,T., Defize,L.H., Angel,P., Woodgett,J.R., Karin,M., and Hunter,T. (1991). *Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity*. Cell *64*, 573-584.

Brown,G.C. (1995). *Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase*. FEBS Lett *369*, 136-139.

Brown,G.C. (1999). *Nitric oxide and mitochondrial respiration*. Biochim. Biophys Acta *1411*, 351-369.

Brown,J.R., Nigh,E., Lee,R.J., Ye,H., Thompson,M.A., Saudou,F., Pestell,R.G., and Greenberg,M.E. (1998). *Fos family members induce cell cycle entry by activating cyclin D1*. Mol Cell Biol *18*, 5609-5619.

Brown,P.H., Chen,T.K., and Birrer,M.J. (1994). *Mechanism of action of a dominant-negative mutant of c-Jun*. Oncogene *9*, 791-799.

Brune,B., von Knethen,A., and Sandau,K.B. (1998). *Nitric oxide and its role in apoptosis*. Eur J Pharmacol *351*, 261-272.

Brune,B., von Knethen,A., and Sandau,K.B. (1999). *Nitric oxide (NO): an effector of apoptosis*. Cell Death. Differ. *6*, 969-975.

Brusselbach,S., Mohle-Steinlein,U., Wang,Z.Q., Schreiber,M., Lucibello,F.C., Muller,R., and Wagner,E.F. (1995). *Cell proliferation and cell cycle progression are not impaired in fibroblasts and ES cells lacking c-Fos*. Oncogene *10*, 79-86.

Bursch,W. (2001). *The autophagosomal-lysosomal compartment in programmed cell death*. Cell Death Differ *8*, 569-581.

Buschmann,T., Potapova,O., Bar-Shira,A., Ivanov,V.N., Fuchs,S.Y., Henderson,S., Fried,V.A., Minamoto,T., Alarcon-Vargas,D., Pincus,M.R., Gaarde,W.A., Holbrook,N.J., Shiloh,Y., and Ronai,Z. (2001). *Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress*. Mol. Cell Biol. *21*, 2743-2754.

Butler,A.R., Flitney,F.W., and Williams,D.L. (1995). *NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective*. Trends Pharmacol Sci *16*, 18-22.

Chakraborty,P. (2001). *G-protein-mediated signaling and its control in macrophages and mammalian cells*. Crit Rev Microbiol *27*, 1-8.

Cheng,A., Chan,S.L., Milhavet,O., Wang,S., and Mattson,M.P. (2001). *p38 MAP kinase mediates nitric oxide-induced apoptosis of neural progenitor cells*. J Biol. Chem. *276*, 43320-43327.

Chernova,O.B., Chernov,M.V., Agarwal,M.L., Taylor,W.R., and Stark,G.R. (1995). *The role of p53 in regulating genomic stability when DNA and RNA synthesis are inhibited*. Trends Biochem Sci *20*, 431-434.

Chiang,L.W., Grenier,J.M., Ettwiller,L., Jenkins,L.P., Ficenec,D., Martin,J., Jin,F., DiStefano,P.S., and Wood,A. (2001). *An orchestrated gene expression component of neuronal programmed cell death revealed by cDNA array analysis*. Proc Natl Acad Sci U S A *98*, 2814-2819.

Chinenov,Y. and Kerppola,T.K. (2001). *Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity*. Oncogene *20*, 2438-2452.

Chou,S.Y., Baichwal,V., and Ferrell,J.E., Jr. (1992). *Inhibition of c-Jun DNA binding by mitogen-activated protein kinase*. Mol Biol Cell *3*, 1117-1130.

Ciesielski-Treska,J., Ulrich,G., Chasserot-Golaz,S., Zwiller,J., Revel,M.O., Aunis,D., and Bader,M.F. (2001). *Mechanisms underlying neuronal death induced by chromogranin A-activated microglia*. J Biol Chem *276*, 13113-13120.

Ciesielski-Treska,J., Ulrich,G., Taupenot,L., Chasserot-Golaz,S., Corti,A., Aunis,D., and Bader,M.F. (1998). *Chromogranin A induces a neurotoxic phenotype in brain microglial cells*. J Biol Chem *273*, 14339-14346.

Claret,F.X., Hibi,M., Dhut,S., Toda,T., and Karin,M. (1996). *A new group of conserved coactivators that increase the specificity of AP-1 transcription factors*. Nature *383*, 453-457.

Colussi,P.A. and Kumar,S. (1999). *Targeted disruption of caspase genes in mice: what they tell us about the functions of individual caspases in apoptosis*. Immunol Cell Biol *77*, 58-63.

Conradt,B. and Horvitz,H.R. (1998). *The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9*. Cell *93*, 519-529.

Cooper,C.E. (1999). *Nitric oxide and iron proteins*. Biochim. Biophys Acta *1411*, 290-309.

Coyle,J.T. and Puttfarcken,P. (1993). *Oxidative stress, glutamate, and neurodegenerative disorders*. Science *262*, 689-695.

Cunningham,B.A., Hemperly,J.J., Murray,B.A., Prediger,E.A., Brackenbury,R., and Edelman,G.M. (1987). *Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing*. Science *236*, 799-806.

Demple,B. (1999). *Genetic responses against nitric oxide toxicity*. Braz. J Med Biol Res *32*, 1417-1427.

Deng,T. and Karin,M. (1994). *c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK*. Nature *371*, 171-175.

Denninger,J.W. and Marletta,M.A. (1999). *Guanylate cyclase and the .NO/cGMP signaling pathway*. Biochim. Biophys Acta *1411*, 334-350.

Desmoucelles,C., Vaudry,H., Eiden,L.E., and Anouar,Y. (1999). *Synergistic action of upstream elements and a promoter-proximal CRE is required for neuroendocrine cell-specific expression and second-messenger regulation of the gene encoding the human secretory protein secretogranin II*. Mol Cell Endocrinol. *157*, 55-66.

Devary,Y., Gottlieb,R.A., Lau,L.F., and Karin,M. (1991). *Rapid and preferential activation of the c-jun gene during the mammalian UV response*. Mol Cell Biol *11*, 2804-2811.

Dhakshinamoorthy,S. and Porter,A.G. (2004). *Nitric oxide-induced transcriptional up-regulation of protective genes by Nrf2 via the antioxidant response element counteracts apoptosis of neuroblastoma cells*. J Biol Chem *279*, 20096-20107.

Dimmeler,S., Haendeler,J., Nehls,M., and Zeiher,A.M. (1997). *Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases*. J Exp Med *185*, 601-607.

Doherty,P. and Walsh,F.S. (1992). *Cell adhesion molecules, second messengers and axonal growth*. Curr Opin Neurobiol. *2*, 595-601.

Ellis,H.M. and Horvitz,H.R. (1986). *Genetic control of programmed cell death in the nematode C. elegans*. Cell *44*, 817-829.

Ellis,R.E., Yuan,J.Y., and Horvitz,H.R. (1991). *Mechanisms and functions of cell death*. Annu Rev Cell Biol *7*, 663-698.

Enoch,T. and Norbury,C. (1995). *Cellular responses to DNA damage: cell-cycle checkpoints, apoptosis and the roles of p53 and ATM*. Trends Biochem Sci *20*, 426-430.

Eom,D.S., Choi,W.S., and Oh,Y.J. (2004). *Bcl-2 enhances neurite extension via activation of c-Jun N-terminal kinase*. Biochem Biophys Res Commun *314*, 377-381.

Estus,S., Zaks,W.J., Freeman,R.S., Gruda,M., Bravo,R., and Johnson,E.M., Jr. (1994). *Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis*. J Cell Biol. *127*, 1717-1727.

Fadok,V.A., Bratton,D.L., Frasch,S.C., Warner,M.L., and Henson,P.M. (1998). *The role of phosphatidylserine in recognition of apoptotic cells by phagocytes*. Cell Death Differ *5*, 551-562.

Fan,M., Goodwin,M., Vu,T., Brantley-Finley,C., Gaarde,W.A., and Chambers,T.C. (2000). *Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade*. J Biol. Chem. *275*, 29980-29985.

Feng,Z., Li,L., Ng,P.Y., and Porter,A.G. (2002). *Neuronal Differentiation and Protection from Nitric Oxide-Induced Apoptosis Require c-Jun-Dependent Expression of NCAM140*. Mol. Cell Biol. *22*, 5357-5366.

Feng,Z. and Porter,A.G. (1999). *NF-kappaB/Rel proteins are required for neuronal differentiation of SH-SY5Y neuroblastoma cells*. J Biol Chem *274*, 30341-30344.

Fischer-Colbrie,R., Laslop,A., and Kirchmair,R. (1995). *Secretogranin II: molecular properties, regulation of biosynthesis and processing to the neuropeptide secretoneurin*. Prog Neurobiol. *46*, 49-70.

Fu,S., Bottoli,I., Goller,M., and Vogt,P.K. (1999). *Heparin-binding epidermal growth factor-like growth factor, a v-Jun target gene, induces oncogenic transformation*. Proc Natl Acad Sci U S A *96*, 5716-5721.

Fujita,Y., Katagi,J., Tabuchi,A., Tsuchiya,T., and Tsuda,M. (1999). *Coactivation of secretogranin-II and BDNF genes mediated by calcium signals in mouse cerebellar granule cells*. Brain Res Mol Brain Res *63*, 316-324.

Fukuto,J.M. (1995). *Chemistry of nitric oxide: biologically relevant aspects*. Adv. Pharmacol *34*, 1-15.

Gasser,M.C., Berti,I., Hauser,K.F., Fischer-Colbrie,R., and Saria,A. (2003). *Secretoneurin promotes pertussis toxin-sensitive neurite outgrowth in cerebellar granule cells*. J Neurochem *85*, 662-669.

Gaston,B. (1999). *Nitric oxide and thiol groups*. Biochim. Biophys Acta *1411*, 323-333.

Ghafourifar,P. and Richter,C. (1997). *Nitric oxide synthase activity in mitochondria*. FEBS Lett *418*, 291-296.

Ghatan,S., Larner,S., Kinoshita,Y., Hetman,M., Patel,L., Xia,Z., Youle,R.J., and Morrison,R.S. (2000). *p38 MAP kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons*. J Cell Biol. *150*, 335-347.

Giudici,A.M., Sher,E., Pelagi,M., Clementi,F., and Zanini,A. (1992). *Immunolocalization of secretogranin II, chromogranin A, and chromogranin B in differentiating human neuroblastoma cells*. Eur J Cell Biol *58*, 383-389.

Giulivi,C., Poderoso,J.J., and Boveris,A. (1998). *Production of nitric oxide by mitochondria*. J Biol Chem *273*, 11038-11043.

Green,D. and Kroemer,G. (1998). *The central executioners of apoptosis: caspases or mitochondria?* Trends Cell Biol *8*, 267-271.

Green,D.R. and Reed,J.C. (1998). *Mitochondria and apoptosis*. Science *281*, 1309-1312.

Grigoriadis,A.E., Schellander,K., Wang,Z.Q., and Wagner,E.F. (1993). *Osteoblasts are target cells for transformation in c-fos transgenic mice*. J Cell Biol *122*, 685-701.

Haas,C.A., Hollerbach,E., Deller,T., Naumann,T., and Frotscher,M. (2000). *Up-regulation of growth-associated protein 43 mRNA in rat medial septum neurons axotomized by fimbria-fornix transection*. Eur. J. Neurosci. *12*, 4233-4242.

Hagn,C., Klein,R.L., Fischer-Colbrie,R., Douglas,B.H., and Winkler,H. (1986). *An immunological characterization of five common antigens of chromaffin granules and of large dense-cored vesicles of sympathetic nerve*. Neurosci. Lett *67*, 295-300.

Hai,T. and Curran,T. (1991). *Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity*. Proc Natl Acad Sci U S A *88*, 3720-3724.

Harada,J. and Sugimoto,M. (1999). *An inhibitor of p38 and JNK MAP kinases prevents activation of caspase and apoptosis of cultured cerebellar granule neurons*. Jpn. J Pharmacol. *79*, 369-378.

Harris,C.A. and Johnson,E.M., Jr. (2001). *BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons*. J Biol. Chem. *276*, 37754-37760.

Hartl,M., Reiter,F., Bader,A.G., Castellazzi,M., and Bister,K. (2001). *JAC, a direct target of oncogenic transcription factor Jun, is involved in cell transformation and tumorigenesis*. Proc Natl Acad Sci U S A *98*, 13601-13606.

Hengartner,M.O. (2000). *The biochemistry of apoptosis*. Nature *407*, 770-776.

Hengartner,M.O., Ellis,R.E., and Horvitz,H.R. (1992). *Caenorhabditis elegans gene ced-9 protects cells from programmed cell death*. Nature *356*, 494-499.

Hengartner,M.O. and Horvitz,H.R. (1994). *C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2*. Cell *76*, 665-676.

Hentze,H., Schmitz,I., Latta,M., Krueger,A., Krammer,P.H., and Wendel,A. (2002). *Glutathione dependence of caspase-8 activation at the death-inducing signaling complex*. J. Biol. Chem. *277*, 5588-5595.

Herdegen,T., Skene,P., and Bahr,M. (1997). *The c-Jun transcription factor--bipotential mediator of neuronal death, survival and regeneration*. Trends Neurosci. *20*, 227-231.

Herdegen,T. and Waetzig,V. (2001). *AP-1 proteins in the adult brain: facts and fiction about effectors of neuroprotection and neurodegeneration*. Oncogene *20*, 2424-2437.

Hilberg,F., Aguzzi,A., Howells,N., and Wagner,E.F. (1993). *c-jun is essential for normal mouse development and hepatogenesis*. Nature *365*, 179-181.

Hilton,M., Middleton,G., and Davies,A.M. (1997). *Bcl-2 influences axonal growth rate in embryonic sensory neurons*. Curr Biol *7*, 798-800.

Hughes,M.N. (1999). *Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite*. Biochim. Biophys Acta *1411*, 263-272.

Hummler,E., Cole,T.J., Blendy,J.A., Ganss,R., Aguzzi,A., Schmid,W., Beermann,F., and Schutz,G. (1994). *Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors*. Proc Natl Acad Sci U S A *91*, 5647-5651.

Ivanov,V.N., Bhoumik,A., Krasilnikov,M., Raz,R., Owen-Schaub,L.B., Levy,D., Horvath,C.M., and Ronai,Z. (2001). *Cooperation between STAT3 and c-jun suppresses Fas transcription*. Mol Cell *7*, 517-528.

Jacobson,M.D., Weil,M., and Raff,M.C. (1997). *Programmed cell death in animal development*. Cell *88*, 347-354.

Jain,J., Nalefski,E.A., McCaffrey,P.G., Johnson,R.S., Spiegelman,B.M., Papaioannou,V., and Rao,A. (1994). *Normal peripheral T-cell function in c-Fos-deficient mice*. Mol Cell Biol *14*, 1566-1574.

Jensen,L.M., Zhang,Y., and Shooter,E.M. (1992). *Steady-state polypeptide modulations associated with nerve growth factor (NGF)-induced terminal differentiation and NGF deprivation-induced apoptosis in human neuroblastoma cells*. J Biol Chem *267*, 19325-19333.

Jochum,W., Passegue,E., and Wagner,E.F. (2001). *AP-1 in mouse development and tumorigenesis*. Oncogene *20*, 2401-2412.

Johnson,R.S., Spiegelman,B.M., and Papaioannou,V. (1992). *Pleiotropic effects of a null mutation in the c-fos proto-oncogene*. Cell *71*, 577-586.

Johnson,R.S., van Lingen,B., Papaioannou,V.E., and Spiegelman,B.M. (1993). *A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture*. Genes Dev *7*, 1309-1317.

Kahler,C.M., Kirchmair,R., Kaufmann,G., Kahler,S.T., Reinisch,N., Fischer-Colbrie,R., Hogue-Angeletti,R., Winkler,H., and Wiedermann,C.J. (1997a). *Inhibition of proliferation and stimulation of migration of endothelial cells by secretoneurin in vitro*. Arterioscler. Thromb. Vasc. Biol *17*, 932-939.

Kahler,C.M., Schratzberger,P., Kaufmann,G., Hochleitner,B., Bechter,O., Gotsch,C., Woll,E., Marschang,P., Herold,M., and Wiedermann,C.J. (2002). *Transendothelial migration of leukocytes and signalling mechanisms in response to the neuropeptide secretoneurin*. Regul. Pept. *105*, 35-46.

Kahler,C.M., Schratzberger,P., and Wiedermann,C.J. (1997b). *Response of vascular smooth muscle cells to the neuropeptide secretoneurin. A functional role for migration and proliferation in vitro*. Arterioscler. Thromb. Vasc. Biol *17*, 2029-2035.

Kallunki,T., Su,B., Tsigelny,I., Sluss,H.K., Derijard,B., Moore,G., Davis,R., and Karin,M. (1994). *JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation*. Genes Dev *8*, 2996-3007.

Kanai,A.J., Pearce,L.L., Clemens,P.R., Birder,L.A., VanBibber,M.M., Choi,S.Y., de Groat,W.C., and Peterson,J. (2001). *Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection*. Proc Natl Acad Sci U S A *98*, 14126-14131.

Kang,D.C., Motwani,M., and Fisher,P.B. (1998). *Role of the transcription factor AP-1 in melanoma differentiation (review)*. Int. J Oncol. *13*, 1117-1126.

Karin,M. (1995). *The regulation of AP-1 activity by mitogen-activated protein kinases*. J Biol Chem *270*, 16483-16486.

Karin,M., Liu,Z., and Zandi,E. (1997). *AP-1 function and regulation*. Curr Opin Cell Biol *9*, 240-246.

Kasibhatla,S., Brunner,T., Genestier,L., Echeverri,F., Mahboubi,A., and Green,D.R. (1998). *DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1*. Mol. Cell *1*, 543-551.

Kataoka,K. and Yanase,H. (1998). *Mild hypothermia--a revived countermeasure against ischemic neuronal damages*. Neurosci. Res. *32*, 103-117.

Kerr,J.F., Wyllie,A.H., and Currie,A.R. (1972). *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics*. Br. J Cancer *26*, 239-257.

Kharbanda,S., Saxena,S., Yoshida,K., Pandey,P., Kaneki,M., Wang,Q., Cheng,K., Chen,Y.N., Campbell,A., Sudha,T., Yuan,Z.M., Narula,J., Weichselbaum,R., Nalin,C., and Kufe,D. (2000). *Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage*. J Biol. Chem. *275*, 322-327.

Kihiko,M.E., Tucker,H.M., Rydel,R.E., and Estus,S. (1999). *c-Jun contributes to amyloid beta-induced neuronal apoptosis but is not necessary for amyloid beta-induced c-jun induction*. J Neurochem. *73*, 2609-2612.

Kim,Y.M., Chung,H.T., Kim,S.S., Han,J.A., Yoo,Y.M., Kim,K.M., Lee,G.H., Yun,H.Y., Green,A., Li,J., Simmons,R.L., and Billiar,T.R. (1999). *Nitric oxide protects PC12 cells from serum deprivation-induced apoptosis by cGMP-dependent inhibition of caspase signaling*. J Neurosci. *19*, 6740-6747.

Kim,Y.M., Chung,H.T., Simmons,R.L., and Billiar,T.R. (2000). *Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase inhibition*. J Biol Chem *275*, 10954-10961.

Kim,Y.M., Kim,T.H., Seol,D.W., Talanian,R.V., and Billiar,T.R. (1998). *Nitric oxide suppression of apoptosis occurs in association with an inhibition of Bcl-2 cleavage and cytochrome c release*. J Biol Chem *273*, 31437-31441.

Kingham,P.J. and Pocock,J.M. (2000). *Microglial apoptosis induced by chromogranin A is mediated by mitochondrial depolarisation and the permeability transition but not by cytochrome c release*. J Neurochem *74*, 1452-1462.

Kolbus,A., Herr,I., Schreiber,M., Debatin,K.M., Wagner,E.F., and Angel,P. (2000). *c-Jun-dependent CD95-L expression is a rate-limiting step in the induction of apoptosis by alkylating agents*. Mol. Cell Biol. *20*, 575-582.

Kroncke,K.D., Fehsel,K., and Kolb-Bachofen,V. (1997). *Nitric oxide: cytotoxicity versus cytoprotection--how, why, when, and where?* Nitric. Oxide. *1*, 107-120.

Landar,A. and Darley-Usmar,V.M. (2003). *Nitric oxide and cell signaling: modulation of redox tone and protein modification*. Amino. Acids *25*, 313-321.

Laslop,A. and Tschernitz,C. (1992). *Effects of nerve growth factor on the biosynthesis of chromogranin A and B, secretogranin II and carboxypeptidase H in rat PC12 cells*. Neuroscience *49*, 443-450.

Le Niculescu,H., Bonfoco,E., Kasuya,Y., Claret,F.X., Green,D.R., and Karin,M. (1999). *Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death*. Mol. Cell Biol. *19*, 751-763.

Le,S., Connors,T.J., and Maroney,A.C. (2001). *c-Jun N-terminal kinase specifically phosphorylates p66ShcA at serine 36 in response to ultraviolet irradiation*. J Biol. Chem. *276*, 48332-48336.

Lei,K. and Davis,R.J. (2003). *JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis*. Proc Natl Acad Sci U S A *100*, 2432-2437.

Leppa,S., Eriksson,M., Saffrich,R., Ansorge,W., and Bohmann,D. (2001). *Complex functions of AP-1 transcription factors in differentiation and survival of PC12 cells*. Mol. Cell Biol. *21*, 4369-4378.

Leppa,S., Saffrich,R., Ansorge,W., and Bohmann,D. (1998). *Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation*. EMBO J *17*, 4404-4413.

Li,J., Billiar,T.R., Talanian,R.V., and Kim,Y.M. (1997). *Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation*. Biochem Biophys Res Commun *240*, 419-424.

Li,J., Lee,J.M., and Johnson,J.A. (2002). *Microarray analysis reveals an antioxidant responsive element-driven gene set involved in conferring protection from an oxidative stress-induced apoptosis in IMR-32 cells*. J Biol Chem *277*, 388-394.

Liebermann,D.A., Gregory,B., and Hoffman,B. (1998). *AP-1 (Fos/Jun) transcription factors in hematopoietic differentiation and apoptosis*. Int. J Oncol. *12*, 685-700.

Lipton,S.A. (1999). *Neuronal protection and destruction by NO*. Cell Death Differ *6*, 943-951.

Lockshin,R.A. and Zakeri,Z. (2004). *Apoptosis, autophagy, and more*. Int. J Biochem Cell Biol *36*, 2405-2419.

Lord,K.A., Abdollahi,A., Hoffman-Liebermann,B., and Liebermann,D.A. (1993). *Proto-oncogenes of the fos/jun family of transcription factors are positive regulators of myeloid differentiation*. Mol Cell Biol *13*, 841-851.

Madsen,M.A., Hsieh,C.C., Boylston,W.H., Flurkey,K., Harrison,D., and Papaconstantinou,J. (2004). *Altered oxidative stress response of the long-lived Snell dwarf mouse*. Biochem Biophys Res Commun *318*, 998-1005.

Mahata,S.K., Mahapatra,N.R., Mahata,M., and O'Connor,D.T. (2002). *Neuroendocrine cell type-specific and inducible expression of chromogranin/secretogranin genes: crucial promoter motifs*. Ann. N Y. Acad Sci *971*, 27-38.

Mahata,S.K., Mahata,M., Livsey,C.V., Gerdes,H.H., Huttner,W.B., and O'Connor,D.T. (1999). *Neuroendocrine cell type-specific and inducible expression of the*

secretogranin II gene: crucial role of cyclic adenosine monophosphate and serum response elements. Endocrinology *140*, 739-749.

Mannick,J.B., Hausladen,A., Liu,L., Hess,D.T., Zeng,M., Miao,Q.X., Kane,L.S., Gow,A.J., and Stamler,J.S. (1999). *Fas-induced caspase denitrosylation*. Science *284*, 651-654.

Mattson,M.P., Culmsee,C., and Yu,Z.F. (2000). *Apoptotic and antiapoptotic mechanisms in stroke*. Cell Tissue Res. *301*, 173-187.

Migliaccio,E., Giorgio,M., Mele,S., Pelicci,G., Reboldi,P., Pandolfi,P.P., Lanfrancone,L., and Pelicci,P.G. (1999). *The p66shc adaptor protein controls oxidative stress response and life span in mammals*. Nature *402*, 309-313.

Min,W., Ghosh,S., and Lengyel,P. (1996). *The interferon-inducible p202 protein as a modulator of transcription: inhibition of NF-kappa B, c-Fos, and c-Jun activities*. Mol Cell Biol *16*, 359-368.

Minden,A., Lin,A., Smeal,T., Derijard,B., Cobb,M., Davis,R., and Karin,M. (1994). *c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases*. Mol Cell Biol *14*, 6683-6688.

Morishima,Y., Gotoh,Y., Zieg,J., Barrett,T., Takano,H., Flavell,R., Davis,R.J., Shirasaki,Y., and Greenberg,M.E. (2001). *Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand*. J Neurosci. *21*, 7551-7560.

Nathan,C. and Xie,Q.W. (1994a). *Nitric oxide synthases: roles, tolls, and controls*. Cell *78*, 915-918.

Nathan,C. and Xie,Q.W. (1994b). *Regulation of biosynthesis of nitric oxide*. J Biol Chem *269*, 13725-13728.

Natori,S. and Huttner,W.B. (1994). *Peptides derived from the granins (chromogranins/secretogranins)*. Biochimie *76*, 277-282.

Oh-Hashi,K., Maruyama,W., Yi,H., Takahashi,T., Naoi,M., and Isobe,K. (1999). *Mitogen-activated protein kinase pathway mediates peroxynitrite-induced apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells*. Biochem. Biophys. Res. Commun. *263*, 504-509.

Palmada,M., Kanwal,S., Rutkoski,N.J., Gustafson-Brown,C., Johnson,R.S., Wisdom,R., Carter,B.D., and Gufstafson-Brown,C. (2002). *c-jun is essential for sympathetic neuronal death induced by NGF withdrawal but not by p75 activation*. J Cell Biol *158*, 453-461.

Passegue,E., Jochum,W., Schorpp-Kistner,M., Mohle-Steinlein,U., and Wagner,E.F. (2001). *Chronic myeloid leukemia with increased granulocyte progenitors in mice lacking junB expression in the myeloid lineage*. Cell *104*, 21-32.

Passegue,E. and Wagner,E.F. (2000). *JunB suppresses cell proliferation by transcriptional activation of p16(INK4a) expression*. EMBO J *19*, 2969-2979.

Pennypacker,K. (1997). *Transcription factors in brain injury*. Histol. Histopathol. *12*, 1125-1133.

Pennypacker,K.R. (1995). *AP-1 transcription factor complexes in CNS disorders and development*. J Fla. Med. Assoc. *82*, 551-554.

Pettmann,B. and Henderson,C.E. (1998). *Neuronal cell death*. Neuron *20*, 633-647.

Potapova,O., Basu,S., Mercola,D., and Holbrook,N.J. (2001). *Protective role for c-Jun in the cellular response to DNA damage*. J Biol. Chem. *276*, 28546-28553.

Pramanik,R., Qi,X., Borowicz,S., Choubey,D., Schultz,R.M., Han,J., and Chen,G. (2003). *p38 isoforms have opposite effects on AP-1-dependent transcription through regulation of c-Jun. The determinant roles of the isoforms in the p38 MAPK signal specificity*. J Biol Chem. *278*, 4831-4839.

Radi,R., Cassina,A., and Hodara,R. (2002). *Nitric oxide and peroxynitrite interactions with mitochondria*. Biol Chem *383*, 401-409.

Raff,M. (1998). *Cell suicide for beginners*. Nature *396*, 119-122.

Raguenez,G., Desire,L., Lantrua,V., and Courtois,Y. (1999). *BCL-2 is upregulated in human SH-SY5Y neuroblastoma cells differentiated by overexpression of fibroblast growth factor 1*. Biochem Biophys Res Commun *258*, 745-751.

Ray,A., Sassone-Corsi,P., and Sehgal,P.B. (1989). *A multiple cytokine- and second messenger-responsive element in the enhancer of the human interleukin-6 gene: similarities with c-fos gene regulation*. Mol Cell Biol *9*, 5537-5547.

Rebollo,A., Dumoutier,L., Renauld,J.C., Zaballos,A., Ayllon,V., and Martinez,A. (2000). *Bcl-3 expression promotes cell survival following interleukin-4 deprivation and is controlled by AP1 and AP1-like transcription factors*. Mol. Cell Biol. *20*, 3407-3416.

Reimold,A.M., Grusby,M.J., Kosaras,B., Fries,J.W., Mori,R., Maniwa,S., Clauss,I.M., Collins,T., Sidman,R.L., Glimcher,M.J., and Glimcher,L.H. (1996). *Chondrodysplasia and neurological abnormalities in ATF-2-deficient mice*. Nature *379*, 262-265.

Rossig,L., Fichtlscherer,B., Breitschopf,K., Haendeler,J., Zeiher,A.M., Mulsch,A., and Dimmeler,S. (1999). *Nitric oxide inhibits caspase-3 by S-nitrosation in vivo*. J Biol Chem *274*, 6823-6826.

Sampayo,J.N., Olsen,A., and Lithgow,G.J. (2003). *Oxidative stress in Caenorhabditis elegans: protective effects of superoxide dismutase/catalase mimetics*. Aging Cell *2*, 319-326.

Saria,A., Troger,J., Kirchmair,R., Fischer-Colbrie,R., Hogue-Angeletti,R., and Winkler,H. (1993). *Secretoneurin releases dopamine from rat striatal slices: a*

biological effect of a peptide derived from secretogranin II (chromogranin C). Neuroscience *54*, 1-4.

Scammell,J.G., Reddy,S., Valentine,D.L., Coker,T.N., Nikolopoulos,S.N., and Ross,R.A. (2000). *Isolation and characterization of the human secretogranin II gene promoter*. Brain Res Mol Brain Res *75*, 8-15.

Schaeffer,H.J. and Weber,M.J. (1999). *Mitogen-activated protein kinases: specific messages from ubiquitous messengers*. Mol Cell Biol *19*, 2435-2444.

Schreiber,M., Kolbus,A., Piu,F., Szabowski,A., Mohle-Steinlein,U., Tian,J., Karin,M., Angel,P., and Wagner,E.F. (1999). *Control of cell cycle progression by c-Jun is p53 dependent*. Genes Dev *13*, 607-619.

Schutte,J., Minna,J.D., and Birrer,M.J. (1989). *Deregulated expression of human c-jun transforms primary rat embryo cells in cooperation with an activated c-Ha-ras gene and transforms rat-1a cells as a single gene*. Proc Natl Acad Sci U S A *86*, 2257-2261.

Shaulian,E. and Karin,M. (2001). *AP-1 in cell proliferation and survival*. Oncogene *20*, 2390-2400.

Shaulian,E. and Karin,M. (2002). *AP-1 as a regulator of cell life and death*. Nat Cell Biol *4*, E131-E136.

Shaulian,E., Schreiber,M., Piu,F., Beeche,M., Wagner,E.F., and Karin,M. (2000). *The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest*. Cell *103*, 897-907.

Slee,E.A., Adrain,C., and Martin,S.J. (1999). *Serial killers: ordering caspase activation events in apoptosis*. Cell Death Differ *6*, 1067-1074.

Smeal,T., Binetruy,B., Mercola,D.A., Birrer,M., and Karin,M. (1991). *Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73*. Nature *354*, 494-496.

Smeyne,R.J., Vendrell,M., Hayward,M., Baker,S.J., Miao,G.G., Schilling,K., Robertson,L.M., Curran,T., and Morgan,J.I. (1993). *Continuous c-fos expression precedes programmed cell death in vivo*. Nature *363*, 166-169.

Steller,H. (1995). *Mechanisms and genes of cellular suicide*. Science *267*, 1445-1449.

Stroh,C. and Schulze-Osthoff,K. (1998). *Death by a thousand cuts: an ever increasing list of caspase substrates*. Cell Death Differ *5*, 997-1000.

Stuehr,D.J. (1999). *Mammalian nitric oxide synthases*. Biochim. Biophys Acta *1411*, 217-230.

Tacke,R. and Goridis,C. (1991). *Alternative splicing in the neural cell adhesion molecule pre-mRNA: regulation of exon 18 skipping depends on the 5'-splice site*. Genes Dev *5*, 1416-1429.

Taupenot,L., Harper,K.L., and O'Connor,D.T. (2003). *The chromogranin-secretogranin family*. N Engl J Med *348*, 1134-1149.

Taylor,D.L., Diemel,L.T., Cuzner,M.L., and Pocock,J.M. (2002). *Activation of group II metabotropic glutamate receptors underlies microglial reactivity and neurotoxicity following stimulation with chromogranin A, a peptide up-regulated in Alzheimer's disease*. J Neurochem *82*, 1179-1191.

Thepot,D., Weitzman,J.B., Barra,J., Segretain,D., Stinnakre,M.G., Babinet,C., and Yaniv,M. (2000). *Targeted disruption of the murine junD gene results in multiple defects in male reproductive function*. Development *127*, 143-153.

Thompson,C.B. (1995). *Apoptosis in the pathogenesis and treatment of disease*. Science *267*, 1456-1462.

Thompson,E.J., Gupta,A., Stratton,M.S., and Bowden,G.T. (2002). *Mechanism of action of a dominant negative c-jun mutant in inhibiting activator protein-1 activation*. Mol. Carcinog. *35*, 157-162.

Tong,L., Toliver-Kinsky,T., Taglialatela,G., Werrbach-Perez,K., Wood,T., and Perez-Polo,J.R. (1998). *Signal transduction in neuronal death*. J Neurochem. *71*, 447-459.

Torreilles,F., Salman-Tabcheh,S., Guerin,M., and Torreilles,J. (1999). *Neurodegenerative disorders: the role of peroxynitrite*. Brain Res. Brain Res. Rev. *30*, 153-163.

Torres,J. and Wilson,M.T. (1999). *The reactions of copper proteins with nitric oxide*. Biochim. Biophys Acta *1411*, 310-322.

Tournier,C., Hess,P., Yang,D.D., Xu,J., Turner,T.K., Nimnual,A., Bar-Sagi,D., Jones,S.N., Flavell,R.A., and Davis,R.J. (2000). *Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway*. Science *288*, 870-874.

van Dam,H., Duyndam,M., Rottier,R., Bosch,A., Vries-Smits,L., Herrlich,P., Zantema,A., Angel,P., and van der Eb,A.J. (1993). *Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein*. EMBO J *12*, 479-487.

Verheij,M., Bose,R., Lin,X.H., Yao,B., Jarvis,W.D., Grant,S., Birrer,M.J., Szabo,E., Zon,L.I., Kyriakis,J.M., Haimovitz-Friedman,A., Fuks,Z., and Kolesnick,R.N. (1996). *Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis*. Nature *380*, 75-79.

von Knethen,A., Callsen,D., and Brune,B. (1999). *NF-kappaB and AP-1 activation by nitric oxide attenuated apoptotic cell death in RAW 264.7 macrophages*. Mol. Biol. Cell *10*, 361-372.

Wang,Z.Q., Ovitt,C., Grigoriadis,A.E., Mohle-Steinlein,U., Ruther,U., and Wagner,E.F. (1992). *Bone and haematopoietic defects in mice lacking c-fos*. Nature *360*, 741-745.

Watson,A., Eilers,A., Lallemand,D., Kyriakis,J., Rubin,L.L., and Ham,J. (1998). *Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons*. J Neurosci. *18*, 751-762.

Weiler,R., Marksteiner,J., Bellmann,R., Wohlfarter,T., Schober,M., Fischer-Colbrie,R., Sperk,G., and Winkler,H. (1990a). *Chromogranins in rat brain: characterization, topographical distribution and regulation of synthesis*. Brain Res *532*, 87-94.

Weiler,R., Meyerson,G., Fischer-Colbrie,R., Laslop,A., Pahlman,S., Floor,E., and Winkler,H. (1990b). *Divergent changes of chromogranin A/secretogranin II levels in differentiating human neuroblastoma cells*. FEBS Lett *265*, 27-29.

Whitfield,J., Neame,S.J., Paquet,L., Bernard,O., and Ham,J. (2001). *Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release*. Neuron *29*, 629-643.

Wiedermann,C.J. (2000). *Secretoneurin: a functional neuropeptide in health and disease*. Peptides *21*, 1289-1298.

Wilson,M.R. (1998). *Apoptosis: unmasking the executioner*. Cell Death Differ *5*, 646-652.

Wisdom,R. (1999). *AP-1: one switch for many signals*. Exp Cell Res *253*, 180-185.

Wisdom,R., Johnson,R.S., and Moore,C. (1999). *c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms*. EMBO J *18*, 188-197.

Wyllie,A.H., Kerr,J.F., and Currie,A.R. (1980). *Cell death: the significance of apoptosis*. Int. Rev Cytol. *68*, 251-306.

Yamagishi,S., Yamada,M., Ishikawa,Y., Matsumoto,T., Ikeuchi,T., and Hatanaka,H. (2001). *p38 mitogen-activated protein kinase regulates low potassium-induced c-Jun phosphorylation and apoptosis in cultured cerebellar granule neurons*. J Biol. Chem. *276*, 5129-5133.

Yamamoto,K., Ichijo,H., and Korsmeyer,S.J. (1999). *BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M*. Mol. Cell Biol. *19*, 8469-8478.

Yang,D.D., Kuan,C.Y., Whitmarsh,A.J., Rincon,M., Zheng,T.S., Davis,R.J., Rakic,P., and Flavell,R.A. (1997). *Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene*. Nature *389*, 865-870.

Yin,K.J., Lee,J.M., Chen,S.D., Xu,J., and Hsu,C.Y. (2002). *Amyloid-beta induces Smac release via AP-1/Bim activation in cerebral endothelial cells*. J Neurosci. *22*, 9764-9770.

Yu,L.Y., Jokitalo,E., Sun,Y.F., Mehlen,P., Lindholm,D., Saarma,M., and Arumae,U. (2003). *GDNF-deprived sympathetic neurons die via a novel nonmitochondrial pathway*. J Cell Biol *163*, 987-997.

Yuan,J., Shaham,S., Ledoux,S., Ellis,H.M., and Horvitz,H.R. (1993). *The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme*. Cell *75*, 641-652.

Yuan,J. and Yankner,B.A. (1999). *Caspase activity sows the seeds of neuronal death*. Nat Cell Biol *1*, E44-E45.

Zou,H., Henzel,W.J., Liu,X., Lutschg,A., and Wang,X. (1997). *Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3*. Cell *90*, 405-413.

Zweier,J.L., Samouilov,A., and Kuppusamy,P. (1999). *Non-enzymatic nitric oxide synthesis in biological systems*. Biochim. Biophys Acta *1411*, 250-262.